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(54) Title: RECOMBINANT DNA-DERIVED CHOLERA TOXIN SUBUNIT ANALOGS

(57) Abstract

The development of subunits and subunit analogs of the cholera exotoxin by recombinant DNA techniques provides vaccine products that can retain their biological activity and immunogenicity, and can confer protection against disease challenge. Genetically-enginneered modifications of the subunits result in products that retain immunogenicity, yet are reduced in, or are essentially free of, enzymatic activity associated with toxin reactogenicity.

RECOMBINANT DNA-DERIVED CHOLERA TOXIN SUBUNIT ANALOGS

BACKGROUND OF THE INVENTION

5 ### Field Of The Invention

The present invention relates to the recombinant expression of analog subunits of cholera exotoxin, and to vaccines based on such analogs. More
10 particularly, genetically engineered modifications of the exotoxin provide analogs of cholera toxin having the capability to elicit a protective response with reduced or essentially no catalytic activity which can contribute to the reactogenicity of cholera vaccines.
15

Description Of The Art

The term "cholera" refers to the disease caused by infection with the etiologic agent *Vibrio*
20 *cholerae*, most commonly occurring in geographical areas where poor hygienic conditions prevail. Cholera remains a major cause of morbidity and mortality in many parts of the world[1,2]. Experience has shown that contraction of the disease usually confers long-
25 lasting protection against subsequent exposure to the etiologic agent[3]. Consequently, considerable effort has been devoted to the development of a vaccine that would be similarly protective. A parenteral whole cell cholera vaccine has been produced, but some no longer
30 regard it as useful, particularly for young children who are at greatest risk from the disease[1].

As for many other infectious diseases, a biological exotoxin (in this case, "cholera toxin" or "CTX") encoded by the genome of the infectious agent
35 and secreted by it, contributes significantly to the ability of the microorganism to colonize the infected

host(4). Moreover, exposure to the toxin causes severe diarrhea and vomiting which result in dehydration, a life-threatening condition of the disease(3,5). These experiences suggest that a vaccine which elicits an immunologic response (e.g., antibodies) sufficient to neutralize the toxin would thus significantly help to prevent or reduce bacterial colonization and attendant symptoms such as diarrhea and vomiting. Thus, substantial effort has been applied toward developing a vaccine containing a non-toxic analog of the toxin, i.e., a "toxoid"(1,3-13). It is known that cholera toxin is a multi-subunit macromolecule consisting of a subunit termed "A", containing a catalytic region called "A1" which ADP-ribosylates G-proteins in target cells, and a "B" oligomer which binds the holotoxin to the target cells(6). Non-toxic analogs of cholera toxin have been produced for purposes of vaccine development by various means. These methods include chemical treatment of the holotoxin or toxin subunits, deletion of the A subunit and use of the remaining B oligomer, and synthesis or isolation of peptide fragments of toxin subunits(1,3-13).

In recent years, efforts have turned toward the development of oral vaccines, with two approaches apparently having received the most attention. One of these approaches is based on the use of killed V. cholerae (i.e., chemically- or heat-inactivated), alone, or supplemented with the B oligomer of cholera toxin(1,11,12). This approach has been found to produce incomplete protection, particularly in young children(12). The other approach involves the use of living, but attenuated, strains of V. cholerae which fail to produce the A1 subunit of the toxin(13). Vaccines of this kind have provided greater levels of protection, but until recently have also been associated with unacceptable intestinal side-effects.

A recently-developed vaccine based on *V. cholerae* strain CVD 103-HgR, in which the gene encoding the A subunit is omitt d, appears to be better tolerated, at least in adults(13). However, to our knowledge,

5 this vaccine has not been tested in children or in large-scale clinical trials.

Recent studies on the nature of cholera toxin have provided insights concerning its structure that may have application in vaccine development based

10 on a recombinant approach. It is known that naturally-ocurring subunit A is synthesized in *V. cholerae* as a preprotein(14), which is subsequently cleaved to proteolytically remove a signal peptide sequence of approximately 2,160 kDa. Further post-translational

15 processing yields an amino-terminal polypeptide of approximately 21,817 kDa (subunit A1) and a carboxyl-terminal polypeptide of approximately 5,398 kDa (subunit A2), which are linked by a disulfide bridge(6,15,16); reduction of the disulfide bond is

20 believed necessary for catalysis of the ADP-ribosyltransferase reaction (6,15,16). Likewise, the B subunit is synthesized as a preprotein which is subsequently cleaved by protease to remove a signal peptide. The genes, or cistronic elements, for the A1,

25 A2 and B subunits of cholera toxin have all been fully sequenced and described in the literature(16).

BRIEF DESCRIPTION OF THE FIGURES

30 FIGURE 1A is the DNA sequence of the cistronic element encoding the A subunit of CTX from the prior art. The single-letter amino acid sequence beneath the DNA sequence indicates the proposed open reading frame for the A polypeptide. Subregions are

35 also indicated, showing the start of the signal peptide (pre-A), A1, two proposed sites for carboxyl-terminal

processing of A1, and th proposed start and
termination of A2. It should be noted that the
literature provides inconclusive vidence as to the
exact location of the carboxyl terminus of A1(16,17).

5

FIGURE 1B is the DNA sequence of the
cistronic element encoding the B subunit of CTX.
Initiation and termination codons and proposed cleavage
sites are likewise shown. Interestingly, the region of
10   DNA in the operon encoding the termination of A2 and
the initiation of B overlap; these two proteins,
however, are in different reading frames.

FIGURE 2 shows schematic structures for the
15   preprotein and processed protein forms of the A and B
subunits of native CTX and the forms of the recombinant
subunits. The "squiggle" at the amino termini of the
preprotein species represents the signal peptide which
is removed by *V. cholerae*. "M" indicates an amino
20   terminal methionine residue; "(M)" indicates that this
is a heterologous (non-native) residue residing at the
amino terminus of the mature recombinant CTXA and CTXA1
subunits, and analogs thereof; amino acid sequence data
indicates that the heterologous methionine residue is
25   not substantially cleaved from the recombinant
polypeptide by cellular methionine amino-peptidase.
"S" indicates the sulfur moiety involved in a disulfide
linkage between cysteine residues. Other selected
amino acids are indicated by their standard single-
30   letter codes, with their position within the
polypeptides indicated. Selected restriction enzyme
cleavage sites for the encoding DNA sequences are
indicated on the encoded polypeptide with their
standard three-letter codes. Native ("n") CTXA is
35   believed to be synthesized in *V. cholerae* as a
preprotein ("pre-A"), containing an amino-terminal

signal sequence. Post-translational processing results in cleavage of the signal to yield mature CTXA. Perhaps simultaneously, a small portion of the carboxyl terminus is also cleaved proteolytically. Th larger A

5   fragment (CTXA1) and the smaller carboxyl-terminal A fragment (CTXA2) are held together after cleavage by a disulfide bridge between the single cysteine residue in each fragment. The literature possesses conflicting reports as to the location of the terminus of CTXA1

10   (either Arg^{192} or Ser^{194}); CTXA2 is believed to begin with Met^{195}. Native ("n") CTXB is also synthesized with an amino-terminal signal sequence that is subsequently processed by protease. Interestingly, the region of the CTXB cistronic element encoding its amino

15   terminus overlaps with the CTXA cistronic element encoding its carboxyl terminus; the coding sequences, however, are in different reading frames(16). Recombinant ("r") CTXA was synthesized in *E. coli* under control of an optimized expression vector. An

20   oligonucleotide linker (NdeI-XbaI) was used for cloning of the left-hand end of the DNA element, substituting an initiating methionine codon for the signal peptide-encoded sequence. The A2 region was not removed from A1 in the recombinant *E. coli*. A similar left-hand

25   cloning strategy was used for CTXB, except an NdeI-AccI fragment was used to substitute the methionine initiation codon for its signal peptide-encoded sequence. Recombinant CTXA1 was synthesized to mimic native, reduced CTXA1. In this regard, an

30   oligonucleotide linker at the right-hand end was used to substitute a termination codon for the A2 sequence such that A1 terminates at Ser^{194}, one of the two proposed cleavage sites in native CTXA1. Termination at Arg^{192} can also be easily accomplished using the

35   same linker strategy. As previously noted, the amino terminal methionines of the recombinant CTXA and CTXA1

molecules, and their analogs, are not believed to be
substantially removed by nascent *E. coli* methionine
aminopeptidase.

5   FIGURE 3 is the SDS-PAGE of native and
recombinant CTX subunits. Recombinant CTXA, CTXA1, the
Arg7→Lys analogs of recombinant CTXA and CTXA1, and
recombinant CTXB were synthesized in *E. coli* and
inclusion bodies prepared as described in the text.
10   The inclusion body preparations, as well as purified
commercial-grade native CTX, CTXA, and CTXB, were
solubilized and subjected to SDS-PAGE under reducing
conditions. Lane 1, native CTX; lane 2, rCTXA/A7;
lane 3, rCTXA Arg7→Lys analog (rCTXA/L7); lane 4,
15   rCTXA1/A7; lane 5, rCTXA1 Arg7→Lys analog (rCTXA1/L7);
lane 6, rCTXB; lane 7, native CTXB; lane 8, native CTXA
(only CTXA1 is visualized). Subsequent to
electrophoresis, the gel was stained with Coomassie
Brilliant Blue R250 and then destained to reveal the
20   stain-retaining polypeptides.

FIGURE 4 is the SDS-PAGE and autoradiographic
analysis of rCTXA1 and CTXA1 analog ADP-
ribosyltransferase activity. In Panel A, native CTXA,
25   recombinant CTXA1, and various site-specific analogs or
preparations of rCTXA1 were subjected to SDS-PAGE and
stained with Coomassie Blue. These same preparations
were used as enzyme sources to ADP-ribosylate membrane-
associated G protein using [32P]NAD under assay
30   conditions described in the text. After the reactions
were quenched, the entire reaction mixture from each
preparation was subjected to SDS-PAGE, and the gel
dried and subjected to autoradiography to visualize
proteins that have been covalently modified by addition
35   of [32P]-labeled ADP-ribose. Panel B shows the result
of the assays when no G-protein substrate was added,

illustrating the ability of recombinant CTXA1 to autoribosylate; interestingly, analog CTXA1/L7 has lost this reactivity. Panel C shows the ADP-ribosylation of substrate G protein found in human erythrocyte

5 membranes. Addition of this substrate substantially shifts reactivity of the enzyme from itself (autoribosylation) to the target G protein (seen in the autoradiogram as its ribosylated α-subunit). Again, rCTXA1 analog L7 lacks this reactivity.

10

FIGURE 5 is the SDS-PAGE and autoradiographic analysis of rCTXA and rCTXA analog ADP-ribosyltransferase activities, similar to that shown for rCTXA1 in Figure 4. Because the rCTXA preparation

15 possesses significantly lower activity than rCTXA1 (see Figure 6), presumably because the former still contains the uncleaved A2 "tail" at its carboxyl terminus, these autoradiograms were attained by a longer exposure of the gel (Panel A) to the x-ray film. Panel A is the

20 stained SDS-polyacrylamide gel of the rCTXA proteins; in comparison with Figure 4, Panel A, it is evident that the recombinant expression of these proteins is generally less than that of the companion rCTXA1 proteins. The recombinant CTXA preparation was capable

25 of autoribosylation (Panel B) and of ADP-ribosylating the G protein substrate in human erythrocyte membranes (Panel C); these activities are substantially diminished in comparison with rCTXA1. Nevertheless, the CTXA preparations exhibit the same general pattern

30 of inactivation as do their CTXA1 counterparts. Again, the L7 analog (Arg7→Lys) is devoid of ADP-ribosylating activity.

FIGURE 6 is the SDS-PAGE and autoradiographic

35 comparison of the ADP-ribosyltransferase activity of rCTXA and rCTXA1/L7 with that of rCTXA1 and rCTXA1/L7.

Panel A is the reactivity without added substrate and
Panel B is with human erythrocyte membranes added as
substrate. The lanes contain: lane 1) blank (no sample
added to reaction); lane 2) native CTXA without urea
5   treatment; lane 3) native CTXA with urea treatment;
lane 4) rCTXA; lane 5) rCTXA/L7; lane 6) rCTXA/L7 plus
native CTXA; lane 7) rCTXA1; lane 8) rCTXA/L7; lane 9)
rCTXA1/L7 plus native CTXA. This experiment
demonstrates that the rCTXA preparation is much less
10   active than rCTXA1 for ADP-ribosylation of G proteins
(compare lanes 4 and 7), yet exhibits substantial
autoribosylating activity. Confirming the data shown
in Figures 4 and 5, substitution of lysine for
arginine-7 in rCTXA and rCTXA1 abolishes their
15   ribosylating activities, both for autocatalysis and for
G protein. Retention of activity by native CTXA when
added to the analog preparations (lanes 6 and 9)
additionally illustrates that it is not a contaminant
of the recombinant preparations that suppress this
20   activity.

FIGURE 7 illustrates the ADP-ribosylation of
H27 fibroblast and erythrocyte membranes by CTXA and
CTXA1 analogs. Naturally-occurring CTXA or
25   recombinant CTXA1 analogs were incubated with [^{32}P]NAD
and either human erythrocyte or H27 fibroblast
membranes. After incubation, the mixtures were
precipitated, centrifuged, and the resulting pellets
subjected to SDS-PAGE. The gels were stained with
30   Coomassie Blue, dried, and subsequently exposed to
x-ray film to produce autoradiograms. B, no CTXA or
CTXA1 analog added; A, naturally-occurring CTXA, A+u,
naturally-occurring CTXA treated with urea; rA1,
recombinant CTXA1 with no residue substitutions; RBC,
35   human erythrocyte membranes.

FIGURE 8 illustrates the ADP-ribosylation of H27 fibroblast and membranes by CTXA and CTXA1 analogs. Naturally-occurring CTXA or recombinant CTXA1 analogs w re incubated with [^{32}P]NAD in th

5 presence of èither human erythrocyte membranes, H27 fibroblast membranes, or no added substrate-containing membranes. After incubation, the mixtures were precipitated, centrifuged, and the resulting pellets were subjected to SDS-PAGE. The gels were stained

10 with Coomassie blue, washed and dried. The upper left panel is a photograph of a stained gel of samples incubated in the absence of substrate-containing membranes; the upper right panel is an autoradiogram of this gel. The lower left and right panels are

15 autoradiograms of gels of samples incubated with erythrocyte and H27 membranes, respectively. B, no CTXA or CTXA1 analog added; A, naturally-occurring CTXA; A + u, naturally-occurring CTXA treated with urea; rA1, recombinant CTXA1 with no residue

20 substitutions; RBC, human erythrocyte membranes.

SUMMARY OF THE INVENTION

The present invention provides a
25 recombinant DNA molecule, at least a portion of which encodes an analog of the catalytic subunit of cholera toxin having reduced enzymatic activity, such activity generally accepted to be associated with vaccine reactogenicity. More specifically, site
30 specific mutagenesis, as described herein, results in analogs of the A and A1 subunits which, compared to the native toxin counterparts, exhibit a significant reduction in catalytic function as measured by ADP-ribosyltransferase activity.

35 The term "catalytic subunit of cholera toxin" used in this disclosure refers to both the

A region of cholera toxin and th Al subregion, as
depicted in Figs. 1A and 2. Thes r gions of the
cholera toxin macromolecule are known to possess ADP-
ribosyltransferase catalytic activity(6). This enzyme
5   is a complex of two sub-activities: an NAD
glycohydrolase activity which cleaves NAD into
nicotinamide and ADP-ribose, and a transferase activity
which transfers the ADP-ribose to the G protein
substrate. Measurements of the ADP-ribosyltransferase
10   activity in this disclosure represent a summation of
both activities. The present invention comprehends
mutagenesized versions of these A and Al polypeptides,
and analogs or derivatives of such polypeptides, which
in their native forms are sources of catalytic activity
15   within the cholera toxin multimer.
          The genetically-engineered analogs of
cholera toxin, which are a product of this invention,
provide recombinant DNA-derived materials suitable for
use in vaccines for the prevention of cholera disease.
20   The A and Al subunit analogs can be used alone or in
combination with B oligomer in a toxoid-based vaccine,
or phenotypically expressed by variants of *V. cholerae,*
or phenotypically expressed under the genetic control
of other immunizing vectors. It should be noted that
25   the analog A and Al subunits of this invention are
utilizable by themselves as antigenic agents in a
vaccine because they may contain important protective
epitopes. However, the use of these analogs in
association with B subunits may be more desirable. The
30   B oligomer contains neutralizing epitopes useful for
eliciting immunoprotection(1,3,5). Association of the
A subunit with the B oligomer may lead to a more
effective immunogenic response against the B oligomer.
The B oligomer can be purified from *V. cholerae* or,
35   alternatively, can be derived recombinantly in a manner
similar to the A and Al subunits by expression in

E. coli or other r combinant hosts, including other bacterial organisms (e.g., *Salmonella typhimurium* or *typhi*, *Bacillus sp.*), yeast (e.g., *S. cerevisiae)*, and viruses (e.g., vaccinia and adenoviruses).

5   Mutagenesis in accordance with this description enables production of mutants varying in diminished catalytic activity, ranging from variants which exhibit attenuated activity to those which are essentially free of such activity (i.e., less than 5%). 10 This flexibility in approach is desirable because attenuation, rather than elimination, of catalytic activity may be helpful in providing a greater degree of and/or longer-lasting, protective response. Moreover, because of their diminished enzymatic 15 activity, the analog subunits provided by this invention are expected to be less reactogenic.

DETAILED DESCRIPTION

20   The present invention provides high-level, direct recombinant expression of all CTX subunits necessary for vaccine production. Further, catalytic subunit analogs provide biological activity that is reduced in, or essentially free of, ADP- 25 ribosyltransferase catalytic activity. The present analogs used alone, or in combination with B oligomer of the toxin (whether derived from natural sources or by recombinant means), can provide products that are useful in a vaccine and greatly reduce the likelihood 30 of side-effects generally accepted to be associated with the catalytic activity in the native toxin. The toxin analogs of the present invention can be formulated into vaccine compositions or used in combination with other immunogenic agents in a multi- 35 component vaccine.

The individual cistronic elements, or portions thereof, encoding th A and B subunits of *V. cholerae* toxin were subcloned and directly expressed individually in a recombinant host cell system

5   (i.e., *E. coli*). In the absence of a native signal peptide (substituted with a methionine to initiate translation), high levels of expression, in the range of 2% to 80% of total cell protein, were obtained. The fermentation of expressor cells resulted in mature

10   species of rCTXA, rCTXA1 and rCTXB, as shown in Fig. 3. It should be noted that rCTXA is not processed to rCTXA1 and rCTXA2 in *E. coli*, presumably due to the absence of the specific enzyme or a failure of rCTXA to be compartmentalized with this enzyme. Thus, rCTXA

15   possesses the A1 sequence covalently linked to the A2 sequence.

Amino acid analysis of selected recombinant molecules demonstrated that the heterologous (non-native) methionyl residue is not

20   substantially removed from the various rCTX and rCTXA1 subunit species by cellular methionine aminopeptidase; thus, these are also methionyl-mature analogs. All of the recombinant proteins were recovered as inclusion bodies from lysed cells. The

25   subunits were found to have migration patterns in reducing SDS-PAGE essentially identical to authentic native subunits, with the exception of rCTXA which is not processed in *E. coli* to result in cleavage of the A2 region from A1. As shown in Fig. 3, high-level

30   recombinant expression of subunits CTXA, CTXA1 and CTXB in *E. coli* was achieved by direct, non-fusion means.

Although alternative methods and materials can be used in the practice of the present invention,

35   the preferred methods and materials are described

below. All references cited hereunder are incorporated herein by reference.

<div align="center">

MATERIALS AND METHODS FOR RECOMBINANT EXPRESSION
OF CTXA, CTXA1 AND CTXB SUBUNITS

</div>

Materials. DNA modifying enzymes were purchased from New England Biolabs (Beverly, MA), Bethesda Research Laboratories (Gaithersburg, MD), Boehringer Mannheim Biochemicals, (Indianapolis, IN), and International Biotechnologies, Inc. (New Haven, CT); enzymes were used according to manufacturer recommendations. All chemicals and biochemicals were analytical reagent grade. Purified, naturally-occurring cholera toxin and toxin subunits were purchased from Sigma Chemical Company (St. Louis, MO) and List Biologicals (Campbell, CA). Synthetic oligonucleotides were synthesized based on methods developed from the chemical procedure of Matteucci and Caruthers(18).

Plasmids and Bacterial Strains. Plasmids pRIT10810 and pRIT10841, (ATCC 39051 and ATCC 39053, respectively), containing the portions of the CTX operon, were obtained from the American Type Culture Collection, Rockville, MD. Expression plasmids pCFM1036, pCFM1146 and pCFM1156 were derived at Amgen.

A description of the expression vector system used herein is described in United States Patent No. 4,710,473 (Morris), which is incorporated herein by reference. Such plasmids contain an inducible promoter, a synthetic ribosome binding site, a cloning cluster, plasmid origin of replication, a transcription terminator, genes for regulating plasmid copy number, and a Kanamycin resistance gene. The derived plasmids differ from each other in a number of respects. The plasmid

pCFM1036 can be derived from pCFM836 (see
U.S. 4,710,473) by substituting the DNA sequence
between the unique AatII and EcoRI restriction sites
containing the synthetic P$_L$ promoter with the
5 following oligonucleotide:

        AatII   EcoRI

        5'-CATCGATTCTAG-3'
10   3'-TGCAGTAGCTAAGATCTTAA-5'

This plasmid contains no inducible promoter preceding
the restriction cluster. The plasmid pCFM1146 can be
derived from pCFM836 by substituting the small DNA
15 sequence between the unique ClaI and XbaI restriction
sites with the following oligonucleotide:

        ClaI   XbaI

20   5'-CGATTTGATT-3'
         3'-TAAACTAAGATC-5'

and by destroying the two endogenous NdeI restriction
sites by end-filling with T4 polymerase enzyme followed
25 by blunt-end ligation. The plasmid contains·no
synthetic ribosome binding site immediately preceding
the restriction cluster. The plasmid pCFM1156 can be
derived from pCFM1146 by substitution of the small DNA
sequence between the unique XbaI and KpnI restriction
30 sites with the following oligonuceotide which installs
an optimized synthetic ribosome binding site:

    XbaI   KpnI

35   5'-CTAGAAGGAAGGAATAACATATGGTTAACGCGTTGGAATTCGGTAC-3'
       3'-TTCCTTCCTTATTGTATACCAATTGCGCAACCTTAAGC-5'

Plasmids pBR322, pUC18, pUC19, and phage M13mp18 and M13mp19 DNA were purchas d from Bethesda Research Laboratories. *E. coli* FM5 cells were derived at Amgen Inc., Thousand Oaks, CA from *E. coli* K-12

5 strain(19) from C.F. Morris and contain the integrated lambda phage repressor gene, CI_{857}(20). Construction of the individual subunit expression plasmids is described herein. Vector production, cell transformation, and colony selection were performed by

10 standard methods(21).

Analytical Procedures. DNA sequencing was done by modification of the primer-extension, chain-termination method(22,23). Protein sequence analyses

15 were performed by automated Edman degradation in an ABI 470A gas-phase microsequenator(24,25) and by standard enzymatic means, the latter to obtain carboxyl-terminal sequences of selected-proteins. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed essentially as

20 described by Laemmli(26), and elution of polypeptides from polyacrylamide gels was similar to the method of Hunkapiller *et al.*(27). The ratio of recombinant protein to total cellular protein or total inclusion body protein was assessed by SDS-PAGE of whole-cell

25 lysates followed by staining with Coomassie Brilliant Blue R250 and subsequent gel scanning by integrative densitometry.

Assays for the measurement of ADP-ribosyltransferase catalytic activity were done

30 as follows: Native CTXA and recombinant subunits were incubated in a solubilization buffer of 8 M urea, 25 mM sodium phosphate (pH 7.0) and 10 mM dithiothreitol (DTT) for one hour at 37°C and centrifuged at 10,000 rpm for 15 minutes without refrigeration. The

35 additions to the solubilization buffer were adjusted to yield 1 μg of native or recombinant A1 per 4 μL, which

was then added to 60 μL of a reaction mixture (see below) and incubated for one hour on ice.

5   Reaction Mixture

Reagent*:	(final)/60 μl	(final)/100 μl
Na_xPO_4, pH 7.0, 1 M	416 mM	250 mM
DTT, 100 mM	5 mM	3 mM
GTP, 10 mM	167 μM	100 μM
Thymidine, 100 mM	17 mM	10 mM
$MgCl_2$, 1 M	5 mM	3 mM
[32P]-NAD	2.5 μCi	2.5 μCi
NAD, 2500 μM	50 μM	30 μM

*The reagents were obtained from commercial sources. Naturally-occurring CTXA was acquired from List Laboratories. As a control, native CTXA was also assayed by incubation in the same buffer as above, but without urea, for 15 minutes at 37°C, then kept on ice until assayed for ADP-ribosyltransferase activity.

Thirty-six μL of water or a buffer containing human erythrocyte membranes(28) were added to yield a final volume of 100 μL for each sample and the samples incubated at 30°C. After 30 minutes, the reaction was terminated by adding 50 μL of 5 mM NAD and 0.03% sodium deoxycholate to each sample and the reaction mixture chilled on ice for 10 minutes. Fifty μL of 40% trichloroacetic acid (TCA) were then added, the samples placed on ice for at least 15 minutes; 2 mL of water were subsequently added to each sample, and the precipitated protein pelleted by centrifugation. The supernatants were removed and the pelleted protein was frozen. On the following day, the pelleted protein was

subjected to SDS-PAGE(26,29). The gel was stained with
Coomassie Brilliant Blue, d stained, dried and
subjected to autoradiography to measure the content of
covalently linked [^{32}P]-labeled ADP-ribose in th

5   proteins of 'the various bands. An approximation of the
specific activities of the recombinant CTXA1 and
recombinant analog CTXA1 proteins (relative to the
activity of native CTXA1) was obtained by densitometric
scanning of the gels and autoradiograms. The stained

10   gels were scanned to approximate the amount of
individual protein added to each reaction mixture. The
autoradiograms were scanned to estimate the amount of
[^{32}P]ADP-ribose transferred to the G protein substrate
as a function of the density of the autoradiographic

15   image.

        Construction of Expression Plasmids. All
expression plasmids were constructed from a series of
E. coli generalized expression vectors differing as
described previously. The individual cholera toxin

20   subunit gene segments were isolated using the
restriction sites shown in Figs. 1 and 2. The
upstream restriction site was just inside the codon
for the amino-terminal residue of the mature,
processed form of the subunit (i.e., without the

25   signal sequence). For purposes of recombinant
expression in E. coli, the portion of the CTX genes
encoding their native signal peptides were deleted and
substituted instead by a methionine initiation codon,
for expression of the "methionyl-mature" form of the

30   subunit analogs. Synthetic oligonucleotide linkers
were employed to effect insertion of the gene segments
into the expression plasmids at an optimal distance
downstream of the synthetic promoter and ribosome
binding site. The upstream linkers restored the

35   reading frame of each gene back to the first codon of

the mature amino t rminus; the oligonucleotides
included a methionyl initiation codon.

Following transformation of *E. coli* FM5
cells with the various plasmid constructs and plating
on Kanamycin-containing agar, appropriate numbers of
colonies were selected, replica-plated, grown as small
liquid cultures ("minipreps"), and induced at 42°C for
4 hours. The minipreps were then screened by light
microscopy for the presence of inclusion bodies in the
bacterial cells. Preparations exhibiting apparent
inclusions were identified and matching colonies from
the replica plates subjected to flask-scale laboratory
fermentation at the induction temperature. Samples
were removed from fermentation at various times post-
induction and examined for the appearance of the
appropriate CTX subunit by SDS-PAGE followed by
Coomassie Brilliant Blue-staining. The structure of
the plasmid from each expression clone was confirmed by
restriction mapping of the isolated plasmid and
verified by DNA sequencing of junction regions.

Expression of Recombinant CTX, CTXA1 and
CTXB. When *E. coli* cells containing, separately, the
CTXA expression plasmid (pCTXA/A7/1156), the CTXA1
expression plasmid (pCTXA1/A7/1156), and the pCTXB
expression plasmid (pCTXB/1156) were fermented at 37°C
and 42°C, they produced major intracellular proteins
(Figure 3) of approximately 27,215 daltons, 21,817
daltons and 11,600 daltons, respectively; recombinant
CTXA1 and CTXB comigrated with authentic (native) CTXA1
and CTXB, respectively, in SDS-PAGE. Our recombinant
CTXA has no native counterpart, since natural CTXA is
cleaved to CTXA1 and CTXA2 by *V. cholerae* protease at
some point before secretion from the organism; A1 and
A2 are held together by a disulfide bond that is
reduced by the buffers used in SDS-PAGE. Partial amino
acid sequence analysis established that recombinant

polypeptide CTXA1/A7 and CTXA1/L7 (see description below) had the amino terminal sequence predicted for the native CTXA1 subunit, but that the heterologous initiating methionine residue is not substantially

5 removed.

Properties of Recombinant CTX Subunits.
Very little, if any, of the CTX subunits appear to be secreted from the *E. coli* cells. The bulk of each subunit was found in the form of inclusion bodies and

10 constituted 2% to 80% of total cellular protein. Cell lysis by French press and low speed centrifugation resulted in pellet fractions that contained up to 80% of their protein as the individual subunits. All the rCTX subunits were detectable in gels stained with

15 Coomassie Brilliant Blue (Figure 3).

CTXA AND CTXA1 ANALOGS

Using techniques of protein engineering and
20 site-specific mutagenesis(19,30), CTXA and CTXA1 analogs were made. From those analogs made and tested by the time of this submission, it was found that mutagenesis of the amino acid residues at positions arginine-7, histidine-44, histidine-70,

25 glutamic acid-112, and aspartic acid-9, and truncation of the carboxyl terminus (at tryptophan-179 of the mature native CTXA sequence) resulted in diminished or essentially no ADP-ribosyltransferase activity.

Construction of the CTXA Expression Plasmid.
30 Plasmid pRIT10841 (ATCC 39053) was cleaved with restriction enzymes XbaI and ClaI and a 552-bp DNA fragment was isolated by gel electrophoresis which contained the left-hand end of the CTXA gene to the region encoding the protease-sensitive portion that

35 results in CTXA cleavage to CTXA1 and CTXA2. Plasmid pRIT10810 (ATCC 39051) was cleaved with restriction

enzymes ClaI and HindII (the latter an isoschizomer of HincII) and a 368-bp DNA fragment was isolated that encoded a portion of the CTXA subunit from the protease-sensitive site (encoded at the ClaI site) (16,17) through the CTXA2 region, past the termination codon of CTXA, and into the alternative open reading frame of the CTXB subunit.

A synthetic oligonucleotide linker was prepared to reconstitute the open reading frame of CTXA from the site encoding the first amino acid of the mature protein sequence (asparagine) to the XbaI site. This linker possessed NdeI cohesiveness at its left-hand end in order to generate a methionine initiation codon that would substitute for the sequence encoding the signal peptide and to facilitate insertion of the gene construction into the expression vector; the right-hand end of the linker possessed an XbaI overlap. This linker possessed the sequence:

5'-TATGAATGATGATAAGTTATATCGGGCAGATT-3'
3'-ACTTACTACTATTCAATATAGCCCGTCTAAGATC-5'

Plasmid pUC19 was digested with NdeI and XbaI and the linker above inserted. After ligation and transformation, a pUC plasmid named p2A/pUC19 was isolated that contained the linker sequence in place of the normal pUC19 NdeI-XbaI sequence.

Plasmid p2A/pUC19 was digested with XbaI and HincII. The large fragment from this digestion was ligated together with the 552-bp XbaI-ClaI DNA fragment containing the left-hand end of the CTXA gene and the 368-bp ClaI-HindII DNA fragment containing the right-hand end of the CTXA gene (past the termination codon and into the alternative open reading frame of the CTXB subunit). This produced a new plasmid containing the

entire mature CTXA gene; this plasmid was called
pCTXA/A7/pUC19.

The *E. coli* expression plasmid pCFM1156 was
digested with NdeI and HindIII to remove this small
5   portion of its cloning cluster. Plasmid pCTXA/A7/pUC19
was also digested with NdeI and HindIII, and a DNA
fragment (772-bp) containing the entire region of the
CTXA gene was isolated. This fragment was subsequently
ligated into the digested pCFM1156 plasmid to produce
10   the CTXA expression plasmid pCTXA/A7/1156. This NdeI-
NdeI fragment could be inserted into pCFM1156 in either
of two orientations, only one of which would produce an
open reading frame giving rise to a large protein when
expressed. This clone was selected (by analysis of
15   induced clones by SDS-PAGE to identify the recombinant
CTXA protein) and the proper orientation confirmed by
DNA sequencing at the upstream NdeI junction region.

Construction of the CTXB Expression Plasmid.

20   Plasmid pRIT10810 (ATCC 39051) was digested with ClaI
and BstXI and a 538-bp DNA fragment was isolated; this
contained the the A2 coding region of CTXA, the entire
CTXB coding region, and a short DNA sequence to the
right of the termination codon of CTXB.
25   A synthetic oligonucleotide linker was
prepared that permitted the cloning of the right-
hand end of the DNA sequence above into pUC19. This
linker possessed BstX1 and HindIII cohesive ends and
had the sequence:
30

          5'-GTGGAATTCGGTACCATGGA-3'
      3'-GAGTCACCTTAAGCCATGGTACCTTCGAA-5'

Plasmid pUC19 was digested with HindIII and
35   AccI (the latter generating a cohesive end compatible
with that generated by ClaI). The large pUC19 fragment

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was ligated with the 538-bp ClaI-BstXI DNA fragment
containing the CTXB and the BstXI-HindIII linker to
produce a plasmid called pCTXB/pUC19. This plasmid was
then digested with HindIII and SspI (the latter just
5   inside the initiation codon for CTXB and downstream
from the ClaI site) to isolate a 345-bP SspI-HindIII
fragment.

        A synthetic oligonucleotide linker was
prepared that possessed NdeI and SspI cohesive ends and
10   the sequence:

                5'-TATGACACCTCAAAAT-3'
                3'-ACTGTGGAGTTTTA-5'

15   Plasmid pCFM1156 was digested with NdeI and
HindIII to remove this portion of its cloning cluster.
The large pCFM1156 DNA fragment was then ligated with
the 345-bp SspI-HindIII fragment containing a portion
of the CTXB gene and the NdeI-SspI linker that restored
20   its left-hand coding region and insinuated a methionine
codon at the left of this coding region to initiate
protein synthesis. The subsequent expression plasmid,
containing the entire CTXB gene with a methionine
initiation codon, was called pCTXB/1156.
25   Linker Mutagenesis. An oligonucleotide
linker called L7 was synthesized to substitute a lysine
codon for that of arginine-7 in CTXA. The sequence of
this linker, with NdeI and XbaI cohesive ends, is shown
in Table 1. The L7 linker was cloned into the NdeI-XbaI
30   site of pUC19 to produce a plasmid called pL7/pUC19.
Plasmid pL7/pUC19 was then digested with XbaI and
HindIII to remove this portion of the pUC19 cloning
cluster and replaced through ligation with the 552-bp
XbaI-ClaI DNA fragment containing the left-hand end of
35   the CTXA gene (see above) and the 368-bp ClaI-HindII
DNA fragment containing the right-hand end of this gene

(see above). This plasmid, called pCTXA/L7/pUC19, was
digested with NdeI, and a 772-bp DNA fragment was
isolated that possessed the entire mature CTXA gene
with a substitution of the arginine-7 codon by a lysine

5   codon. Plasmid pCFM1156 was digested with NdeI and
ligated with the NdeI DNA fragment from pCTXA/L7/pUC19.
This ligation produced a plasmid called pCTXA/L7/1156
for expression of the mature form of an $Arg^7 \rightarrow Lys$
analog of CTXA in *E. coli*. As with the case of

10 pCTXA/A7/1156 (above), it was necessary to select a
clone containing this plasmid with the DNA insert in
the proper open reading frame for synthesis of
rCTXA/L7.

　　　　Oligonucleotide linkers 1E and 1F were

15 synthesized to individually substitute, respectively, a
phenylalanine codon for that of tyrosine-6 and a
glutamate codon for that of aspartate-9. These linkers
possessed NdeI and XbaI cohesive ends and had the
sequences shown in Table 1. Plasmid pCTXA/A7/pUC19 (see

20 above) was digested with XbaI and HindIII, and a 938-bp
DNA fragment containing the right-hand portion of the
CTXA gene was isolated. Plasmid pCFM1156 was digested
with NdeI and HindIII to remove this short region of
its cloning cluster. This segment was replaced by

25 ligation with the NdeI-XbaI linker containing either
the $Tyr^6 \rightarrow Phe$ or the $Asp^9 \rightarrow Glu$ codon mutation (linkers
1E and 1F, respectively) and the 938-bp DNA fragment of
the CTXA gene. This produced two plasmids,
pCTXA/1E/1156 and pCTXA/1F/1156, for expression of the

30 mature forms of the CTXA analogs $Tyr^6 \rightarrow Phe$ and
$Asp^9 \rightarrow Glu$, respectively, in *E. coli*.

　　　　The substitutions of sequences encoding
mutations of glutamine for proline-185 and alanine for
cysteine-187 resulted in CTXA gene fragments encoding

35 only the CTXA1 portion of the CTXA subunit (see below
for construction of the native-sequence CTXA1 gene and

the L7, 1E, and 1F substitution analogs of CTXA1 from
the CTXA gene and its substitution analogs,
respectively). Oligonucleotide linkers 1G and 1H were
synthesized to individually substitute, respectively,

5 glutamine for proline-185 and alanine for cysteine-
187. These linkers had DsaI and HindIII cohesive ends
and possessed the sequences shown in Table 1. To
effect the construction of the expression plasmids
encoding the analog proteins, a 537-bp NdeI-DsaI DNA

10 fragment was isolated from plasmid pCTXA/A7/pUC19.
Plasmid pCFM1156 was then digested with NdeI and
HindIII to remove this short segment of its cloning
cluster. This segment was replaced by ligation with
the 537-bp DNA fragment from pCTXA/A7/pUC19 and either

15 1G or 1H synthetic oligonucleotides. The linkers, in
addition to encoding the specific amino acid
substitutions, eliminate from the CTXA gene that
portion encoding the A2 region of the CTXA subunit;
thus, these mutations are exclusively in CTXA1

20 versions of the subunit. The resulting plasmids for
expression of the Pro^{185}→Gln and Cys^{187}→Ala analogs of
CTXA1 were called pCTXA1/1G/1156 and pCTXA1/1H/1156,
respectively.

A plasmid expressing a carboxyl-terminal

25 truncated version of CTXA1 terminating at Trp^{179} was
constructed. This was accomplished by first digesting
plasmid pCFM1156 with NdeI and HindIII to remove this
short DNA fragment. Into this site in pCFM1156 was
ligated the 537-bp NdeI-DsaI fragment from

30 pCTXA/A7/PUC19 (see above) and a synthetic DNA fragment
with DsaI and HindIII cohesive ends, and having the
sequence:

5'-CGTGGTAATGATAGA-3'
35   3'-CATTACTATCTTCGA-5'

This plasmid, for expression of CTXA1 truncated at Trp^{179}, was called pCTXA1/T1/1156.

<u>Mutagenesis By Site-directed Priming</u>.

Mutagen sis by site-directed priming was accomplished
5   with kits of the "Altered Sites™ *in vitro* Mutagenesis System" purchased from Promega Corporation (Madison, WI); details of the experimental protocols for this procedure are contained in the technical manual available from Promega Corporation (printed 1/90).

10   To facilitate mutagenesis, a 938-bp XbaI-HindIII DNA fragment encoding a portion of the CTXA subunit was isolated from plasmid pCTXA/A7/pUC19 (see above). This fragment was cloned into the pSELECT1 phagemid vector from Promega. After packaging with
15   helper phage, this vector contained a negative-sense copy of the CTXA fragment. A series of single-stranded, positive-sense DNA primers were synthesized to effect mutagenesis; the sequences of these primers (1B, 1C, 1D, and 1I) are shown in Table 1. These primers were
20   individually annealed with the single-stranded phagemid containing the CTXA gene fragment; double-stranded phagemids were subsequently produced which contained the gene fragment and the individual codon substitutions encoded by the primers.

25   For preparation of plasmids capable of expressing the CTXA and CTXA1 subunit analogs containing a lysine substitution for arginine-146, a 207-bp BstXI-ClaI DNA fragment was isolated from the double-stranded phagemid containing the Arg^{146}→Lys
30   codon mutation (1I). A 375-bp NdeI-BstXI DNA fragment and a 386-bp ClaI-HindIII fragment (for the CTXA version) containing a portion of the CTXA gene were isolated from plasmid pCTXA/A7/pUC19. Plasmid pCFM1156 was digested with NdeI and HindIII to remove this
35   short portion of its cloning cluster. For construction of the CTXA version of the Arg^{146}→Lys mutation, the

digested pCFM1156 plasmid was ligated with the 375-bp
NdeI-BstXI fragm nt from pCTXA/A7/pUC19, the 209-bp
BstXI-ClaI fragment from the double-stranded phagemid,
and th 386-bp ClaI-HindIII DNA fragment from

5   pCTXA/A7/pUC19. This resulted in a plasmid called
pCTXA/1I/1156 for expression of the Arg^{146}→Lys analog
of the CTXA subunit in *E. coli*. For construction of
this mutation in the CTXA1 version of the subunit, the
digested pCFM1156 plasmid was ligated with the 375-bp

10   NdeI-BstXI fragment from pCTXA/A7/pUC19, the 209-bp
BstXI-ClaI fragment isolated from the double-stranded
phagemid, and a synthetic oligonucleotide linker that
replaces a region of CTXA encoding the A2 portion of
CTXA with a DNA sequence encoding the end of the A1

15   region and including a codon that terminates
polypeptide synthesis at the end of CTXA1. This linker
possessed ClaI and HindIII cohesive ends and had the
sequence:

20   5′ CGTAATAGGCGGCCGCA-3′
                      3′-ATTATCCGCCGGCGTCGA-5′

        The resultant plasmid for expression of the
Arg^{146}→Lys analog of CTXA1 in *E. coli* was called

25   pCTXA1/1I/1156.
        Preparation of plasmids capable of
expressing individual analogs of CTXA containing the
substitutions of His^{44}→Asn, His^{70}→Asn, or Glu^{112}→Gln
was facilitated with primers (1B, 1C, and 1D,

30   respectively) having the sequences shown in Table 1.
After annealing of the primers individually to the
pSELECT1 phagemid containing the 938-bp XbaI-HindIII
CTXA fragment from pCTXA/A7/pUC19 (see above) and
recovering double-stranded plasmid, the regions

35   containing the site-specific mutations were excised
from the plasmid by digesting with XbaI and HindIII,

and recovering a 938-bp DNA fragment in each case.
Plasmid p2A/pUC19 (containing an NdeI-XbaI linker
encoding the left-hand end of the mature CTXA; see
above) was digested with XbaI and HindIII to remove
5   this short region of the pUC19 cloning cluster to the
right of the linker insert; this region was replaced
by ligation with the 938-bp XbaI-HindIII fragment
from the plasmid containing a single codon
replacement. This series of pUC-derived plasmids were
10   called pCTXA/1B/pUC19, pCTXA/1C/pUC19, and
pCTXA/1D/pUC19, depending upon the codon replacement
they contained. A DNA fragment containing the codon
replacement was subsequently excised from each of
these plasmids. Plasmid CTXA/A7/pUC19 was digested
15   with BstXI and HindIII and a 593-bp DNA fragment was
isolated. Plasmid pCFM1156 was digested with NdeI
and HindIII to remove this short region of its
cloning cluster, as described earlier, and this
replaced by ligation with the individual CTXA analog
20   gene inserts recovered from the pUC transition
plasmids above and the 593-bp BstXI-HindIII DNA
fragment from pCTX/A7/pUC19. When isolated, these
new plasmids for expression of the site-specific
analogs His^{44}→Asn, His^{70}→Asn, and Glu^{112}→Gln of CTXA
25   in *E. coli* were called pCTXA/1B/1156, pCTXA/1C/1156,
and pCTXA/1D/1156, respectively.

Conversion of CTXA and CTXA Analog Genes to
CTXA1 and CTXA1 Analog Genes. With the exception of
30   the plasmid containing the 1I codon substitution
(pCTXA1/1I/1156), which was constructed during the
mutagenesis process to lack the A2-encoding region, it
was useful to convert the CTXA gene-containing and
selected individual analog gene-containing expression
35   plasmids to CTXA1 expression plasmids in order to
express the A1 truncated version of CTXA that mimicked

the native species of this subunit in r duced
holotoxin preparations. To perform this conversion, it
was necessary to delete a portion of the gene sequence
of the CTXA gene (and the analog genes) to the right

5   of the unique ClaI site. Although the actual site of
polypeptide cleavage between the A1 and A2 regions has
not been resolved in the prior art literature(16,17),
it was decided to initially establish the carboxyl
terminus of A1 at serine-194; it should be noted,

10   however, that establishing the terminus at arginine-
192 (the other terminus proposed in the literature) is
a simple matter of inserting a new linker to
substitute a termination codon immediately to the
right of the arginine-192 codon.

15   For our purposes, each of the analog CTXA
sequences (and the native CTXA sequence) we wished to
convert to CTXA1 versions were excised from their pUC19
transition plasmids (i.e., pCTXA/A7/pUC19,
pCTXA/1B/pUC19, pCTXA/1C/pUC19, pCTXA/1D/pUC19,

20   pCTXA/1E/pUC19, pCTXA/1F/pUC19, pCTXA/1G/pUC19,
pCTXA/1H/pUC19) with restriction enzymes NdeI (at the
sequence encoding the methionine initiation codon) and
ClaI (at the site chosen for addition of a termination
codon immediately to the right of the serine-194

25   codon); this DNA fragment was 585-bp in each·case. For
purposes of substituting a termination codon for the
A2-encoding region and subsequent ligation of the gene
segments into plasmid pCFM1156, an oligonucleotide
linker was synthesized to possess ClaI and HindIII

30   cohesive ends and had the following sequence:

                5'-CGTAATAGGCGGCCGCA-3'
                 3'-ATTATCCGCCGGCGTTCGA-5'

35   Plasmid pCFM1156 was digested with NdeI and
HindIII to remove this portion of its cloning cluster;

this region was replaced by ligation with the ClaI-
HindIII linker and with an individual 585-bp DNA
fragment from one of the pUC transition plasmids
described abov . Isolation of plasmid DNA following
these ligations resulted in a series of plasmids
capable of expressing CTXA1 and CTXA1 analog
polypeptides in *E. coli*; plasmids prepared in this
manner included pCTXA1/1B/1156, pCTXA1/1C/1156,
pCTXA1/1D/1156, pCTXA1/1E/1156, and pCTXA1/1F/1156,.

**Expression and Analysis of CTXA and
Recombinant Analogs**. Following preparation, each
plasmid was used to transform a separate preparation of
fresh, competent FM5 cells. Transformants were picked,
grown as minipreps, induced to produce recombinant
protein, and inclusion body-positive samples identified
by light microscopy. These samples were fermented at a
larger scale ($\geq$ 1 liter) at the induction temperature
to prepare greater amounts of each recombinant analog
protein. Isolated cell pastes were lysed in a French
press after resuspension in distilled H_2O with 1 mM
DTT. Inclusion bodies were isolated from these lysates
by simple low-speed centrifugation. These inclusion-
body protein preparations contained as little as 2% and
as much as 80% of the recombinant proteins. The
samples were assessed for ADP-ribosyltransferase
activity as previously described. The results obtained
are shown in Figs. 4, 5, and 6 and in Table 2.

TABLE 1
CONSTRUCTION OF S1 ANALOGS

*	MUTATION	TECHNIQUE	OLIGONUCLEOTIDE SEQUENCE
L7	ARG7->Lys	Linker Insertion	5'-TATGAATGATGATAAGTTATATAAGGCAGATT-3' 3'-ACTTACTACTATTCAATATATTCCGTCTAAGATC-5'
1B	His44->Asn	Site-directed Priming	5'-CCTTTATGATAACGCAAGAGGAA-3'
1C	His70->Asn	Site-directed Priming	5'GAGAAGTGCCAACTTAGTGGGTC-3'
1D	Glu112->Gln	Site-directed Priming	5'-AGATGAACAACAGGTTTCTGCTT-3'
1E	Tyr6->Phe	Linker Insertion	5'-TATGAATGATGATAAGTTATTCGGGCAGATT-3' 3'-ACTTACTACTATTCAATAAGCCCGTCTAAGATC-5'
1F	Asp9->Glu	Linker Insertion	5'-TATGAATGATGATAAGTTATATGGGCAGAAT-3' 3'-ACTTACTACTATTCAATATAGCCCGTCTTAGATC-5'
1G	Pro185->Gln	Linker Insertion	5'-CGTGGATTCATCATGCACCGCAGGGTTGTGGGAATGCTCCAAGATCATCGTAGA-3' 3'-CTAAGTAGTACGTGGCGTCCCAACACCCTTACGAGGTTCTAGTAGCATCTTCGA-5'
1H	Cys187->Ala	Linker Insertion	5'-CGTGGATTCATCATGCACCGCCGGGTGCAGGGAATGCTCCAAGATCATCGTAGA-3' 3'-CTAAGTAGTACGTGGCGGCCCACGTCCCTTACGAGGTTCTAGTAGCATCTTCGA-5'
1I	Arg146->Lys	Site-directed Priming	5'-GGGGCTACAAGGATAGATAT-3'
T1	COOH Truncation @ Trp179	Linker Insertion	5'-CGTGGGTAAATGATAGA-3' 3'-CATTACTATCTTCGA-5'

*Designation

TABLE 2

ADP-RIBOSYLTRANSFERASE ACTIVITIES OF RECOMBINANT CTXA1 ANALOGS[1]

CTX MOLECULE	MUTATION	PROTEIN ADDED TO ASSAY(mG)[2]	SPECIFIC ACTIVTY FOR HEM G. PROTEIN[3]
Commercial CTXA1 (without urea)	none	1.00	1.00
Commercial CTXA1 (with urea)	none	1.11	0.53
rCTXA1/A7	none	1.56	0.56
rCTXA1/L7	Arg7->Lys	1.46	0
rCTXA1/1B	His44->Asn	1.47	0
rCTXA1/1C	His70->Asn	1.51	0.05
rCTXA1/1D	Glu112->Gln	1.65	0
rCTXA1/1E	Tyr6->Phe	1.04	1.01
rCTXA1/1F	Asp9->Glu	0.91	0.10
rCTXA1/1G	Pro185->Gln	1.23	0.81
rCTXA1/1H	Cys187->Ala	1.14	0.83
rCTXA1/1I	Arg146->Lys	1.05	0.83
rCTXA1/T1	Truncated at Trp179	1.85	<0.01

1The absolute amount of each protein used in each ADP-ribosyltransferase assay (see Figure 4) was estimated by densitometric scanning of the stained SDS-polyacrylamide gel (Figure 4, panel A) containing identical amounts of each protein used in the assay. The autoradiogram of the gel containing the human erythrocyte membranes (Figure 4, Panel C) was subsequently scanned to determine the radiographic density of the G protein alpha subunit ribosylated by each CTXA1 protein preparation. The density of the G protein band resulting from ADP-ribosylation with commercial CTXA1 without added urea was taken as 1.00 and the density of the band resulting form ribosylation by the other CTXA1 proteins was related to this preparation as a percentage of its density. These fractions were then normalized to 1.00 μg of added CTXA1 protein based on the densitometric of the stained gel to obtain an approximate, relative specific activity.

2The amount of commercial CTXA1 (without added urea) in the assay was taken as 1.00 μg.

3The radiographic density of the G protein alpha subunit ADP-ribosylated by the commercial CTXA1 (without added urea) was taken as 1.00.

Figure 4 shows a stained SDS-polyacrylamide gel (Panel A) of inclusion-body preparations of rCTXA1 and its site-specific analogs. An amount of protein identical to that shown in this gel was used to

5 catalyze the individual ADP-ribosyltransferase reactions. Trichloroacetic acid (TCA) precipitates from these reactions were also run in SDS-PAGE and the gels subjected to autoradiography to illuminate the [^{32}P]ADP-ribose-labeled substrates. Panel B

10 illustrates the results of the reactions without added G protein-containing human erythrocyte membrane preparation and Panel C shows the reactions with this added substrate.

The most important finding of these

15 experiments is found in Figure 4, Panel C (and confirmed in Panel B): certain site-specific amino acid residue substitutions result in diminishment and, in some cases, apparently complete loss of enzyme activity as measured in this assay. In this regard,

20 rCTXA1/L7 (Arg^9→Lys), rCTXA1/1B (His^{44}→Asn) and rCTXA1/1D (Glu^{112}→Gln) analog subunits appear to possess virtually no enzyme activity, whereas analogs rCTXA1/1C (His^{70}→Asn) and rCTXA1/1F (Asp^9→Glu) appear to have reduced activity when compared with both native

25 CTXA (with urea) and rCTX1/A7 (no mutation other than the methionine residue at the amino terminus). Truncation at Trp^{179} (rCTXA1/T1/1156) also results in an analog A subunit with severely diminished enzyme activity.

30 Although these autoradiographic assays of enzyme activity are not strictly quantitative, we have attempted to derive a quantitative assessment from the gel and autoradiograms of Figure 4 to illustrate in a numerical sense what can be visually observed. This

35 evaluation is found in Table 2. Here, we subjected the stained SDS-polyacrylamide gel (Fig. 4, Panel A),

containing rCTXA1 and each of the analogs described
previously, to integrative scanning densitometry to
more accurately assess the relative amount of each
protein added to the assay; these were related to the

5   amount of A1 subunit in native CTXA (without urea)
added to the assay, taken as a value of 1.00 µg.
Although an attempt was made to add equivalent amounts
of each protein to the assays (estimated on the basis
of the percentage of subunit protein in each inclusion

10   body preparation), it can be seen that this estimation
may have lacked precision. The autoradiogram of the
subsequent enzyme reactions with G protein substrate
(Fig. 4, Panel C) was also subjected to densitometry to
determine the relative density of the radiographic

15   image of the radiolabeled G protein α subunit band with
that labeled by native CTXA (no urea) taken as 100%.
An approximate-relative specific activity was then
calculated by dividing the image density by the amount
of added enzyme, with the specific activity of native

20   CTX (without urea) taken as 1.00. It should be noted
that the results of this type of quantitation are
subject to certain experimental limitations (e.g.,
assumption of equal dye staining by each of the subunit
preparations, band selection and circumscription for

25   digitized densitometry, densitometer response
characteristics, and assumption of a linear
relationship between [^{32}P]ADP-ribose labelling and
radiographic density). Nevertheless, the results
(Table 2) illustrate in a numerical manner what can be

30   visually observed in the autoradiograms: marked
diminishment of enzyme activity in analogs rCTXA1/1C
(His^{70}→Asn), rCTXA1/1F (Asp^{9}→Glu), and rCTXA1/T1(Trp^{179}
truncation) and virtual loss of activity by analogs
rCTXA1/L7 (Arg^{9}→Lsy), rCTXA1/1B (His^{44}→Asn), and

35   rCTXA1/1D (Glu^{112}→Gln).

In the case in which no exogenous substrate
is added (Figure 4, Panel B), both native CTXA and the
enzymatically-active CTXA1 proteins can be seen to be
autocatalytic, i.e., to catalyze the hydrolysis of NAD
5   and the transfer of ADP-ribose to the enzyme itself
(either in *cis*, in *trans*, or both). Multiple bands
seen in the autoradiogram may be due to contaminating
E. coli proteins capable of being ADP-ribosylated;
alternatively, yet unlikely, they may represent minor
10   variants of the subunit proteins (e.g.,
proteolytically-nicked or, perhaps, variants possessing
some residual secondary structure in SDS). Recombinant
CTXA1 preparations appear much more capable of
participating in the autocatalytic process than does
15   the A subunit of native CTX. The reasons for this
increased autoribosylation are not presently
understood, although it may be related to lack of
substrate specificity by the yet-to-be-renatured
recombinant protein, exposure of a sensitive
20   ribosylation site in the recombinant protein as a
result of improper secondary structure (no attempt was
made in this particular experiment to achieve native
conformation), or to the presence of ARFs (ADP-
ribosylation factors) (31-37) in the crude recombinant
25   preparations that stabilize the autocatalysis.
However, when G protein substrate is added in the form
of human erythrocyte membranes (Panel C), the focus of
the ADP-ribosyltransferase reaction is shifted to this
substrate, quenching autoribosylation.
30   Figure 5 demonstrates that the same general
pattern of diminishment and loss of enzyme activity
seen with the rCTXA1 analogs is also observed when the
same residue substitutions are made in rCTXA versions
of the recombinant subunit (i.e., versions with the A2
35   "tail" still covalently linked). However, the presence
of the A2 region appears to significantly reduce the

ADP-ribosyltransferase of the enzymatically-active
proteins. This reduction is more clearly illustrated
in Figure 6, in which identical amounts of rCTXA and
rCTXA1 are evaluated in the enzyme assay (Panel A), the
5   radiolabeled products run on the same gel, and
consequently subjected to equivalent autoradiographic
exposure times (Panel B). As can be seen, rCTXA1
appears to possess greater activity than rCTXA (compare
lanes 7 and 4). Again, neither subunit construction
10   with the $Arg^9 \rightarrow Lys$ substitution (lanes 5 and 8) possess
measurable ADP-ribosyltransferase activity for the
G protein substrate. That this loss of enzyme activity
in the analogs is not the result of *E. coli*
contaminants suppressing catalysis is evident by the
15   ability of native CTXA to ribosylate G protein in the
presence of the *E. coli*-produced, analog-containing
preparations (lanes 6 and 9).

        Because of their reduction or essential
elimination of a major marker of toxic activity (ADP-
20   ribosyltransferase), the recombinant CTXA1 analog
molecules produced by clones pCTXA1/L7/1156,
pCTXA1/1B/1156, pCTXA1/1C/1156, pCTXA1/1D/1156,
pCTXA1/1F/1156, and pCTXA1/T1/1156, as well as their
rCTXA analog counterparts, are anticipated to have
25   application alone or in combination with CTXB in safer
vaccines. The described mutations would not be
expected to reduce the normal, protective, immunogenic
properties of native CTX subunits. The CTXA and CTXA1
analogs of this invention thus have application in
30   combination with CTXB subunits in the form of a
holotoxoid. The CTXB subunits may augment the immune
response to CTXA and CTXA1, and vice-versa, and each
may have protective epitopes. The CTXB subunits can
be derived from *V. cholerae* or can be genetically-
35   engineered subunits and their analogs. Genetically-

engineered subunit products can include fusion
proteins and non-fusion proteins.

Strategies identical to those described
above were employed to prepare additional recombinant
5   analogs of the CTXA subunit of cholera toxin.   The
synthetic oligonucleotides utilized to effect codon
substitutions, whether by linker mutagenesis or by
mutagenesis by site-directed priming, are shown in
Table 3.   Briefly, analog CTXA1/1J (Asp$^9 \rightarrow$Tyr) was
10   prepared by linker mutagenesis as described for analog
CTXA1/1F (Asp$^9 \rightarrow$Glu), with the exception that the
synthetic oligonucleotide possessed the appropriate
codon substitution.   For the construction of analogs
CTXA1/1K (Ser$^{10} \rightarrow$Gly), CTXA1/1L (Arg$^{11} \rightarrow$Lys), and
15   CTXA1/1M (Arg$^{11} \rightarrow$His), a novel DraII (also known as
EcoO109I) restriction site was introduced into the
CTXA1 gene by site-directed priming utilizing the
following synthetic oligonucleotide primer:

20   5'-AGCAGTCAGGGGGCCTTATGCCAA-3'

Introduction of this site permitted linker mutagenesis
in this region of the gene (using the previously-
described NdeI site to the left of the insertion site
25   and the newly-created DraII site to the right of the
insertion site) to effect the codon changes that
resulted in these three analogs.   Site-directed
priming was the method used to create the codon
changes resulting in analogs CTXA1/1N (His$^{44} \rightarrow$Tyr),
30   CTXA1/1"O" (His$^{44} \rightarrow$Gln), CTXA1/1P (His$^{44} \rightarrow$Val), CTXA1/1Q
(His$^{70} \rightarrow$Tyr), CTXA1/1R (His$^{70} \rightarrow$Gln), and CTXA1/1S
(His$^{70} \rightarrow$Val).

With two exceptions, each of these analogs
was expressed in recombinant *E. coli* and the isolated
35   inclusion bodies were tested for their enzymatic
ability to ADP-ribosylate either G$_s\alpha$ in human

erythrocyte membrane pr parations or, especially in
the case of the His[44] and His[70] analogs, their ability
to ADP-ribosylate $G_s\alpha$ and/or tubulin in membrane
preparations of H27 cultured human foreskin

5   fibroblasts Yprovided by the University of California,
San Francisco). The exceptions were for analog
CTXA1/1J (Asp[9]→Tyr), which was recombinantly expressed
but not assayed for activity, and analog CTXA1/1L
(Arg[11]→Lys), for which a linker had been synthesized

10   and cloning performed, but for which a correct-
sequence clone had not been isolated.

       The results of these analyses are presented
in Figures 4 and 7, and are summarized in Table 4,
Figure 4 provides comparative data for analogs

15   reported in Table 1. Among the additional analogs
described in Figure 7 and Table 4 are three having
different substitutions at His[44] (CTXA1/1N,
CTXA1/1"O", CTXA1/1P). The absence of measurable
enzyme activity in these analogs, in addition to lack

20   of activity in previously-described analog CTXA1/1B
(His[44]→Asn), indicates that these specific
substitutions at His[44] lead to inactivation of the
subunit of cholera toxin possessing the intrinsic
toxic activity of the multimeric molecule. Based on

25   these results, it is likely that *any* substitution at
this residue will produce such inactivation.

       Three analogs (CTXA1/1Q, CTXA1/1R, CTXA1/1S)
having substitutions for His[70] are also among those
described. These analogs are in addition to the

30   analog CTXA1/1C (His[70]→Asn) of Table 1. As shown in
Figure 7, all four His[70] analogs possess *reduced*
ability to ADP-ribosylate $G_s\alpha$ substrate, although they
clearly *retain* the ability to ADP-ribosylate other
non-$G_s\alpha$ protein substrates (e.g., tubulin in H27

35   fibroblasts). Thus, substitutions for His[70] result in
apparent reduction of activity of CTXA1 for the

specific $G_s\alpha$ substrate believed to be involved in the pathognomonic cytotoxic response to cholera toxin. Such substitutions, if made in CTXA1 involved in a formed holotoxin multimer, would therefore likely

5   result in an' attenuated cholera toxin molecule as opposed to one totally lacking toxic properties.

Analysis of two additional analogs is shown in Figure 8. CTXA1/1K (Ser[10]→Gly) retains the catalytic activity associated with the native CTXA

10   molecule. Substitution of His for Arg[11] (CTXA1/1M) results in an analog having little or no measurable enzymatic activity. It would be expected that analog CTXA1/1L (Arg[11]→Lys) will also have significantly diminished activity when isolated and assayed, a

15   conclusion which is supported by the findings of Table 1, (see Arg[7]→Lys).

20

25

TABLE 3

CONSTRUCTION OF CTXA1 ANALOGS

ANALOG	MUTATION	TECHNIQUE
CTXA1/1J	Asp9->Tyr	Linker Insertion ,
CTXA1/1K	Ser10->Gly	Linker Insertion
CTXA1/1L	Arg11->Lys	Linker Insertion
CTXA1/1M	Arg11->His	Linker Insertion
CTXA1/1N	His44->Tyr	Site-directed Priming
CTXA1/1"O"	His44->Gln	Site-directed Priming
CTXA1/1P	His44->Val	Site-directed Priming
CTXA1/1Q	His70->Tyr	Site-directed Priming
CTXA1/1R	His70->Gln	Site-directed Priming
CTXA1/1S	His70->Val	Site-directed Priming

ANALOG	OLIGONUCLEOTIDE SEQUENCE
CTXA1/1J	TAT GAA TGA TGA TAA GTT ATA TCG GGC ATA CT A CTT ACT ATT CAA TAT AGC CCG TAT GAG ATC
CTXA1/1K	TAT GAA TGA TGA TAA GTT ATA TCG GGC AGA TGG CAG ACC TCC TGA TGA AAT AAA GCA GTC AGG G A CTT ACT ATT CAA TAT AGC CCG TCT ACC GTC TGG AGG ACT ACT TTA TTT CGT CAG TCC CCC G
CTXA1/1L	TAT GAA TGA TGA TAA GTT ATA TCG GGC AGA TTC TAA GCC TCC TGA TGA AAT AAA GCA GTC AGG G A CTT ACT ATT CAA TAT AGC CCG TCT AAG ATT CGG AGG ACT ACT TTA TTT CGT CAG TCC CCC G
CTXA1/1M	TAT GAA TGA TGA TAA GTT ATA TCG GGC AGA TTC TCA CCC TCC TGA TGA AAT AAA GCA GTC AGG G A CTT ACT ATT CAA TAT AGC CCG TCT AAG AGT GGG AGG ACT ACT TTA TTT CGT CAG TCC CCC G
CTXA1/1N	CTT TAT GAT TAC GCA AGA GGA
CTXA1/1"O"	CTT TAT GAT CAG GCA AGA GGA
CTXA1/1P	CTT TAT GAT GTT GCA AGA GGA
CTXA1/1Q	AGA AGT GCC TAC TTA GTG GGT
CTXA1/1R	AGA AGT GCC CAG TTA GTG GGT
CTXA1/1S	AGA AGT GCC GTT TTA GTG GGT

TABLE 4

ADP-Ribosyltransferase Activities of CTXA1 Analogs

ANALOG	MUTATION	ADP-ribosyltransferase Activity [1]
CTXA1/1J	Asp9->Tyr	N.D.
CTXA1/1K	Ser10->Gly	[+]
CTXA1/1L	Arg11->Lys	N.D.
CTXA1/1M	Arg11->His	[-]
CTXA1/1N	His44->Tyr	[-]
CTXA1/1"O"	His44->Gln	[-]
CTXA1/1P	His44->Val	[-]
CTXA1/1Q	His70->Tyr	[±]
CTXA1/1R	His70->Gln	[±]
CTXA1/1S	His70->Val	[±]

[1] As visualized by SDS-PAGE and autoradiography: [+], full activity; [±], reduced activity; [-], no detectable activity; N.D., not determined.

IN VITRO ASSOCIATION OF rCTX SUBUNITS

A number of methods by which native cholera
toxin can be dissociated and the individual subunits
5   reassociated *in vitro* to reform the holotoxin molecules
have been described in the literature(36,37). *In vitro*
reassociation of the subunits of pertussis toxin has
also been described in the literature for native
subunits(38-40). Using a similar procedure,
10   recombinant CTX subunits can be isolated, associated
in vitro to form holotoxin-like species, and purified.
In general, following expression and recovery, the
individual subunits are combined in stoichiometric
ratios (based on their relative content of specific
15   subunit protein, if in the form of inclusion body
preparations), approximating the ratio of subunits
found in native CTX holotoxin. The preparation is
solubilized in an aqueous solution containing a
chaotropic agent or a detergent, or both. The
20   preparation is subjected to reducing conditions
(generally a reducing agent or a hydrogen atmosphere,
or both) and then oxidized (with either an oxidizing
agent or under an oxygen-enriched atmosphere, or both)
to reform the necessary intramolecular disulfide
25   bridges. Association of the subunits into holotoxin-
like species is accomplished by diminishment or removal
of the chaotropic or detergent solubilizing agent.
This can be accomplished by a variety of means, to
include filtration and buffer exchange by dialysis
30   chromatography. The holotoxin-like species are then
purified by conventional means, e.g., ion exchange,
size-exclusion and affinity chromatography. It should
be noted that B multimeric species, without the
A subunit, may be recovered by similar means if
35   inclusion-body preparations of the latter subunit are
not added.

The genetically engineered analog subunits
of this invention can be formulated, in a conventional
manner, into a toxoided cholera vaccin . In the case
of a toxin that has been "genetically" inactivated,

5 such as cholera toxin in the present invention, further
inactivating steps (such as chemical treatment or heat
treatment) should not usually be required since these
products are produced in non-pathogenic organisms and
are inherently free of the enzyme activities that are

10 generally accepted to elicit the adverse reactions to
whole-cell cholera vaccines. Nevertheless, it is
necessary to control purity of the recombinant product,
particularly with regard to the endotoxin content. In
general, recombinant holotoxoid, recombinant

15 holotoxoid-like macromolecules, recombinant B subunit
macromolecules, recombinant B subunit alone or possibly
B subunit recombinant analogs, and even A subunit
analogs alone described in the present invention as
potential vaccinating antigens would be purified to

20 $\geq 90\%$ homogeneity. The nature and estimated quantity of
contaminants, if any, would be evaluated to ensure that
the extent of endotoxin contamination meets the
standards of the individual regulatory agencies.

         For purposes of parenteral delivery, the

25 vaccine materials would normally be adsorbed onto
aluminum adjuvants. This can be accomplished by at
least two means: precipitation with preformed alum and
precipitation with aluminum salts. The adsorbed
precipitates are then resuspended in an excipient to

30 yield a dosage concentration of vaccine antigen
generally in the range of 5-100 μg per dose and an alum
amount usually not exceeding 1.5 mg/dose; volume per
dose is in the range of 0.1-1.0 ml. The suspending
excipient is commonly a buffered solution (e.g.,

35 phosphate-buffered saline, pH 7.0), may have added
stabilizers (e.g., glycerol), and will likely contain a

preservative (e.g., 0.01% Thimerosol) to prevent microbial contamination and to extend shelf lif .

The formulation and deliv ry of recombinant cholera toxoid, or subcomponents thereof, *via* live vector systems as also encompassed within this invention will depend upon the nature of that system. For example, oral delivery of recombinant (mutant) *V. cholerae*, *Salmonella sp.*, vaccinia virus, or adenovirus carrying genes for the A or A *and* B subunits, might well be encapsulated in enteric-coated delivery vehicles for passage to the gut or in aerosolizable forms (e.g., with liposomes) for targeting to the respiratory tract in order to elicit secretory immunoglobulin A antibodies for protection at mucosal surfaces. Alternatively, other oral forms of the vaccine can be prepared in accordance with procedures described in the literature, suitably adapted to accommodate the present antigenic agents. For instance, a recombinant *V. cholerae* strain can be lyophilized and mixed with a bicarbonate buffer to neutralize gastric acidity(41); or a holotoxoid in accordance with this invention can be used in the form of an effervescent tablet, appropriately buffered, to supplement a killed, whole-cell vaccine(1).

*   *   *

- 44 -

While this invention has been specifically
illustrated in relation to recombinant production in
E. coli, it will be appreciated by those skilled in the
art that the principles for mutagenesis of the analog
5   subunits as described herein may be employed in
connection with other recombinant hosts and expression
systems, and to produce other inactivated analogs of
the toxin. Further, it should be understood that
assembly of mutant analogs into a holotoxoid can take
10   place in intact cells via homologous recombination,
e.g., in *V. cholerae*, rather than *in vitro*. It is
intended that the present invention include all
modifications and improvements as come within the scope
of the present invention as claimed.

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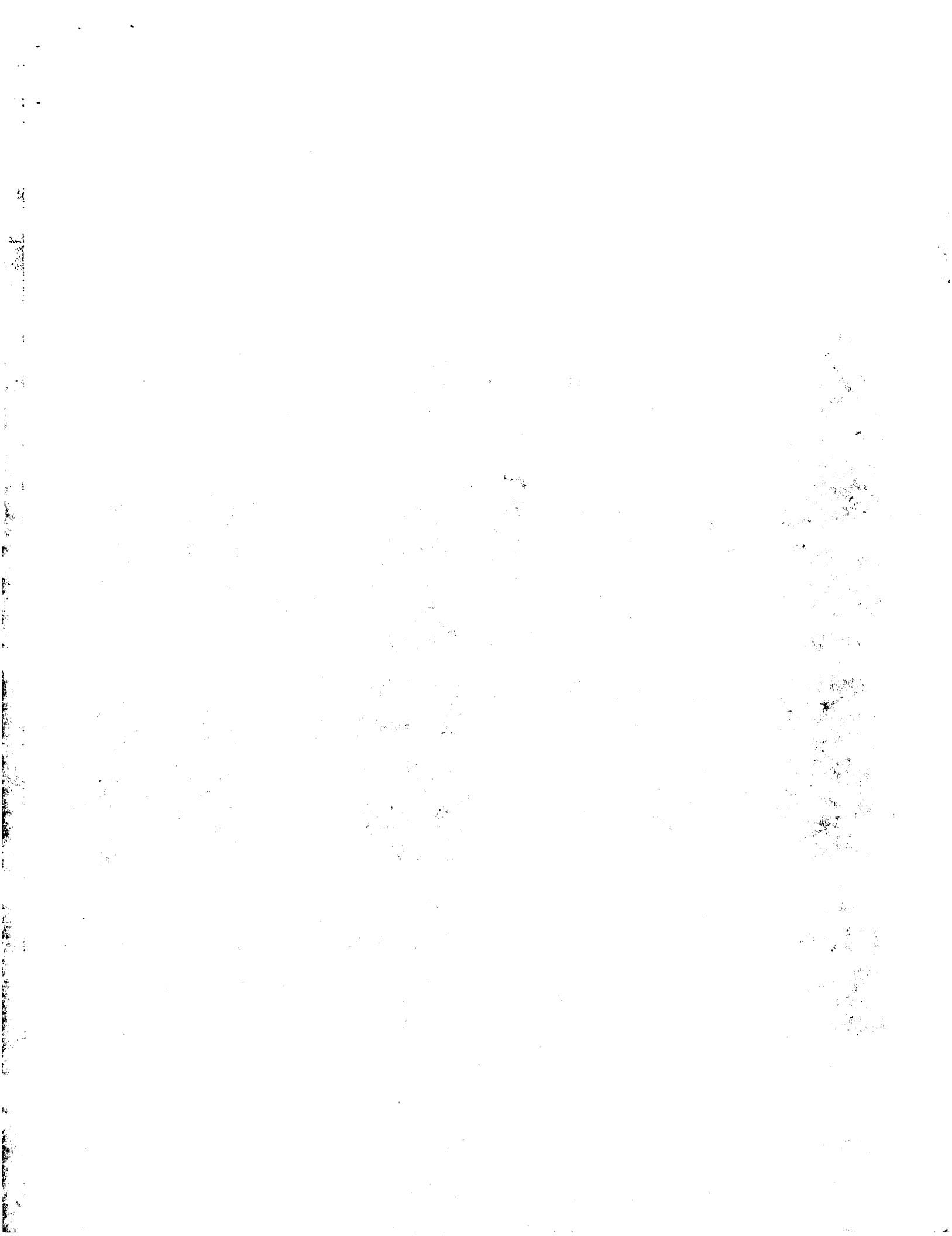11. Black, R.E. et al. (1987) *Infect. Immun.*
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8. The recombinant DNA molecule of
claim 6, which encodes an analog of the catalytic
subunit comprising a truncation of the carboxyl-
terminal portion beginning at tryptophan-179.

5

9. The recombinant DNA molecule of
claim 1, which also encodes subunit B of cholera toxin.

10. A genetically engineered analog of the
catalytic subunit of cholera toxin, said analog having
reduced or essentially no catalytic activity associated
with cholera toxin reactogenicity.

11. The analog of claim 10, which is of the
A region of cholera toxin.

12. The analog of claim 10, which is of the
A1 subunit of cholera toxin.

13. The analog of claim 10, which is
capable of eliciting a cholera toxin-neutralizing
immune response.

14. The analog of claim 10, which is
obtained by site-specific mutagenesis resulting in a
mutation of the catalytic subunit which is less active
or essentially inactive as determined by assay of ADP-
ribosyltransferase activity.

15. The analog of claim 14, wherein the
site-specific mutation is in the region bounded by
methionine-1 and arginine-192 or serine-194,
inclusively.

16. Th analog of claim 15, which comprises a site-sp cific mutation in one or more of the sites of said subunit selected from among arginine-7, arginine-11, aspartic acid-9, histidine-44,

5 histidine-70 and glutamic acid-112.

17. The analog of claim 15, which comprises a truncation of the carboxyl-terminal portion beginning at tryptophan-179.

10

18. An improved anti-cholera vaccine comprising an effective amount of an analog of the catalytic subunit of cholera toxin, wherein said toxin has a biological activity which (a) can elicit a

15 cholera toxin-neutralizing immune response and (b) has reduced or essentially no catalytic activity associated with cholera toxin reactogenicity.

19. The improved vaccine of claim 18,

20 wherein the analog is of the A region of cholera toxin.

20. The improved vaccine of claim 18, wherein the analog is of the A1 subunit of cholera toxin.

25

21. The improved vaccine of claim 18, wherein the toxin-neutralizing immune response provides immunoprotection against cholera disease.

30 22. The improved vaccine of claim 18, wherein the analog has been derived by site-specific mutagenesis resulting in a mutation of the catalytic subunit of cholera toxin which has less or essentially no ADP-ribosyltransferase activity.

35

23. The improved vaccine of claim 22, wherein th site-specific mutation is in the region of the catalytic subunit bounded by methionine-1 and arginine-192 or serine-194, inclusively.

24. The improved vaccine of claim 23, wherein the site-specific mutation is in one or more of the sites of said subunit selected from among arginine-7, arginine-11, aspartic acid-9, histidine-44, histidine-70, and glutamic acid-112.

25. The improved vaccine of claim 23, wherein the mutation comprises truncation of the carboxyl-terminal portion beginning at tryptophan-179.

26. The improved vaccine of claim 18, wherein the analog of the catalytic subunit is associated with the B oligomer.

27. The improved vaccine of claim 26, wherein the B oligomer is the native form.

28. The improved vaccine of claim 26, wherein the B oligomer has been genetically engineered.

29. A prokaryotic or eukaryotic cell transformed with a DNA molecule according to claim 1 which is capable of expressing the polypeptide product or products encoded by said DNA molecule.

30. An *E. coli* host cell according to claim 29.

31. A strain of *Vibrio cholerae* containing a genetically engineered toxin operon in which one or more specific amino acid residues responsible for cholera toxin reactogenicity have been altered or
5 deleted by mutagenesis.

32. The strain of *Vibrio cholerae* according to claim 31, wherein the operon has been mutagenized to alter one or more amino acids selected from among
10 arginine-7, arginine-11, aspartic acid-9, histidine-44, histidine-70, and glutamic acid-112, or to truncate the carboxyl terminal portion beginning at tryptophan-179.

33. A method for the production of an
15 analog of the catalytic subunit of cholera toxin which has reduced or essentially no catalytic activity associated with cholera toxin reactogenicity, comprising:

(a) identifying one or more amino acid
20 residues of the toxin which are associated with such catalytic activity;

(b) effecting site-directed mutagenesis of the toxin cistron or operon to remove or replace such residue or residues and produce a mutagenized cistron
25 or operon, and

(c) expressing the mutagenized cistron or operon in a transformed organism to produce a toxoid characterized by reduced or essentially no catalytic activity.
30

34. An isolated, genetically engineered protein having substantially the amino acid sequence of Figure 1A starting with asparagine-1 of the mature sequence and ending with leucine-240.
35

35. The protein of claim 34 which has been derived by expression in a host other than *Vibrio cholerae*.

5   36. The protein of claim 35 which has been expressed in *E. coli*.

→ preA

→ mature A

1   ATGGTAAAGATAAATATTTGTGTTTTTATTTTCTTATCATCATTTTCATATGCAAAATGAT 60
    TACCATTTCTATTTATAAACACAAAAAATAAAGAATAGTAGTAAAAGTATACGTTTACTA
    M V K I F F V F F I F L S S F S Y A N D

    Ssp I   Nde I
                   Xba I

61 GATAAGTTATATCGGGCCAGATTCTAGACCTCCTGATGAAATAAAGCAGTCAGGTGGTCTT 120
    CTATTCAATATAGCCCGGTCTAAGATCTGGAGGACTACTTTATTTCGTCAGTCCACCAGAA
    D K L Y R A D S R P P D E I K Q S G G L

121 ATGCCAAGAGGACAGAGTGAGTACTTTGACCGAGGTACTCAAATGAATATCAACCTTTAT 180
    TACGGTTCTCCTGTCTCACTCATGAAACTGGCTCCATGAGTTTACTTATAGTTGGAAATA
    M P R G Q S E Y F D R G T Q M N I N L Y

    Sca I   Taq I   Bcl I

181 GATCATGCAAGAGGAACTCAGACGGGGATTTGTTAGGCACGATGATGGATATGTTTCCACC 240
    CTAGTACGTTCTCCTTGAGTCTGCCCCTAAACAATCCGTGCTACTACCTATACAAAGGTGG
    D H A R G T Q T G F V R H D D G Y V S T

FIG. IA

```
B
s        D
p        rP
1        af
2        Il
8        IM
6        II
I        /

241 TCAATTAGTTTGAGAGAAGTGCCCACTTAGTGGGTCAAACTATATTGTCTGGTCATTCTACT 300
    AGTTAATCAAACTCTTCACGGGTGAATCACCCAGTTTGATATAACAGACCAGTAAGATGA -
     S   I   S   L   R   S   A   H   L   V   G   Q   T   I   L   S   G   H   S   T   -

         A         N         T
         f         s         a
         l         p         q
         I         H         I
         I         I         I

301 TATTATATATATGTTTATAGCCCACTGCACCCAACATGTTTAACGTTAAGTGATGTATTAGGG 360
    ATAATATATACAATATCGGTGACGTGGGTTGTACAAATTGCAATTACTACATAATCCC -
     Y   Y   I   Y   V   I   A   T   A   P   N   M   F   N   V   N   D   V   L   G   -

361 GCATACAGTCCCTCATCCAGATGAACAAGAAGTTTCTGCTTTAGGTGGGATTCCATACTCC 420
    CGTATGTCAGGAGTAGGTCTCTACTTGTTCTTCAAAGACGAAATCCACCCTAAGGTATGAGG -
     A   Y   S   P   H   P   D   E   Q   E   V   S   A   L   G   G   I   P   Y   S   -
```

FIG. IA CONT.'

```
                                                                        B
                                                                        s
                                                                        p
                                                                        M
                                                                        I
421 CAAATATATGGATGGTATCGAGTTCATTTGGGGTGCTTGATGAACAATTACATCGTAAT·480
     Q  I  Y  G  W  Y  R  V  H  F  G  V  L  D  E  Q  L  H  R  N  -
  B                                                 G           T
  s                                                 s           t
  t                                                 u           h
  X                                                 I           1
  I                                                             1
                                                                1
                                                                I
                                                                I
    GTTTATATACCTACCATAGCTCAAGTAAAACCCCACGAACTACTTGTTAATGTAGCATTA

                                                                K   B
                                                                s   s
                                                                p   p
                                                                6   M
                                                                3   I
                                                                2   I
                                                                I
481 AGGGGCTACAGAGATATTACAGTAACTTAGATATTGCTCCAGCAGCAGATGGTTAT 540
     R  G  Y  R  D  R  Y  Y  S  N  L  D  I  A  P  A  A  D  G  Y  -
    TCCCCGATGTCTCTATCTCTATAATGTCATTGAATCTATAACGAGGTCGTCGTCTACCAATA

                                                            D
                                                            s
                                                            a
                                                            I
541 GGATTGGCAGGTTTCCCTCCCGGAGCATAGAGCTTGGAGGGAAGAGCCGTGGATTCATCAT 600
     G  L  A  G  F  P  P  E  H  R  A  W  R  E  E  P  W  I  H  H  -
    CCTAACCGTCCAAAGGGAGGCCTCGTATCTCGAACCTCCCTTCTCGGCACCTAAGTAGTA
```

FIG. 1A cont'

second proposed
B authentic C
s COOH   l
m end   a   start
            I   A2
         I A1

```
601  GCACCGCCCGGGGTTGTGTGGGAATGCCTCCAAGATCATCGATGAGTAATACTTGCGATGAAAAA  660
     CGTGGCGGGCCCAACACCCTTACGAGGTTCTAGTAGCTACTCATTATGAACGCTACTTTT
      A  P  P  G  C  G  N  A  P  R  S  S  M  S  N  T  C  D  E  K
```

first proposed
authentic COOH
end and recombinant
end of A1

```
661  ACCCAAAGTCTAGGTGTAAAATTCCTGACGAATACCAATCTAAAGTTAAAAGACAAATA  720
     TGGGTTTCAGATCCCACATTTTAAGGAACTGCTTATGGTTAGATTTCAATTTTCTGTTTAT
      T  Q  S  L  G  V  K  F  L  D  E  Y  Q  S  K  V  K  R  Q  I
```

S   S
s   s
p   p
I   I

End A2

```
721  TTTTCAGGCTATCAATCTGATATTGATACACATAATAGAATTAAGGATGAATTATGA  777
     AAAAGTCCGATAGTTAGACTATAACTATGTGTTATTATCTTAATTCCTACTTAATACT
      F  S  G  Y  Q  S  D  I  D  T  H  N  R  I  K  D  E  L  *
```

FIG. IA CONT.'

```
          EcoRI
           57

     ─>PreB
   1 ATGATTAAAATTAAAAATTTGGTGTGTTTTTTTTACTATCTTCAGCATATGCACAT   -
     TACTAATTTAATTTTTAAACCACACAAAAAAAATGATAGAAGTCGTATACGTGTA  60
       M  I  K  L  K  F  G  V  F  F  T  V  L  L  S  S  A  Y  A  T

        S
        s
        p
        B
        I
     ─>Mature
  61 GGAACACCTCAAAATATTACTGATTTGTGTGCAGAATACCACACACAAATATATACG   -
     CCTTGTGGAGTTTTATAATGACTAAACACACGTCTTATGGTGTGTTTATATATGC 120
       G  T  P  Q  N  I  T  D  L  C  A  E  Y  H  N  T  Q  I  Y  T

                          AX
                          cc
                          ca
                          II
 121 CTAAATGATAAGATATTTCGTATACAGAATCTCTAGCTGGAAAAAGAGAGATGGCTATC  -
     GATTTACTATTCTATAAAGCATATGTCTTAGAGATCGACCTTTTCTCTCTACCGATAG 180
       L  N  D  K  I  F  S  Y  T  E  S  L  A  G  K  R  E  M  A  I
```

FIG. 1B

```
181 ATTACTTTTAAGAATGGTGCAATTTTTCAAGTAGTAGAAGTACCAAGTAGTCAACATATAGAT 240
    TAATGAAAATTCTTACCACGTTAAAAAGTTCATCTTCCAGTTGTTCATCAGTTGTATATCTA
    I  F  K  N  G  A  I  F  Q  V  E  V  P  S  S  Q  H  I  D  .   -
                                                            Hinc II
```

```
241 TCACAAAAAAAAGCGATTGAAAGGATGAAGGATACCCTGAGGATTGCATATCTTACTGAA 300
    AGTGTTTTTTTTCGCTAACTTTCCTACTTCCTATGGGACTCCTAACGTATAGAATGACTT
    S  Q  K  K  A  I  E  R  M  K  D  T  L  R  I  A  Y  L  T  E   -
                                       Eco57I                  Eco57I
```

```
301 GCTAAAGTCGAAAAGTTATGTGTATGGAATAATAAAACGCCTCATGCGATTGCCGCAATT 360
    CGATTTCAGCTTTTTCAATACACATACCTTATTATTTTGCGGAGTACGCTAACGGCGTTAA
    A  K  V  E  K  L  C  V  W  N  N  K  T  P  H  A  I  A  A  I   -
             End B ⤶
```

```
361 AGTATGGCAAATTAA 375
    TCATACCGTTTAATT
    S  M  A  N  *   -
```

FIG. 1B cont.

DEFINITION AND RESTRICTION MAPS OF CHOLERA TOXIN
A AND B SUBUNITS

FIG. 2
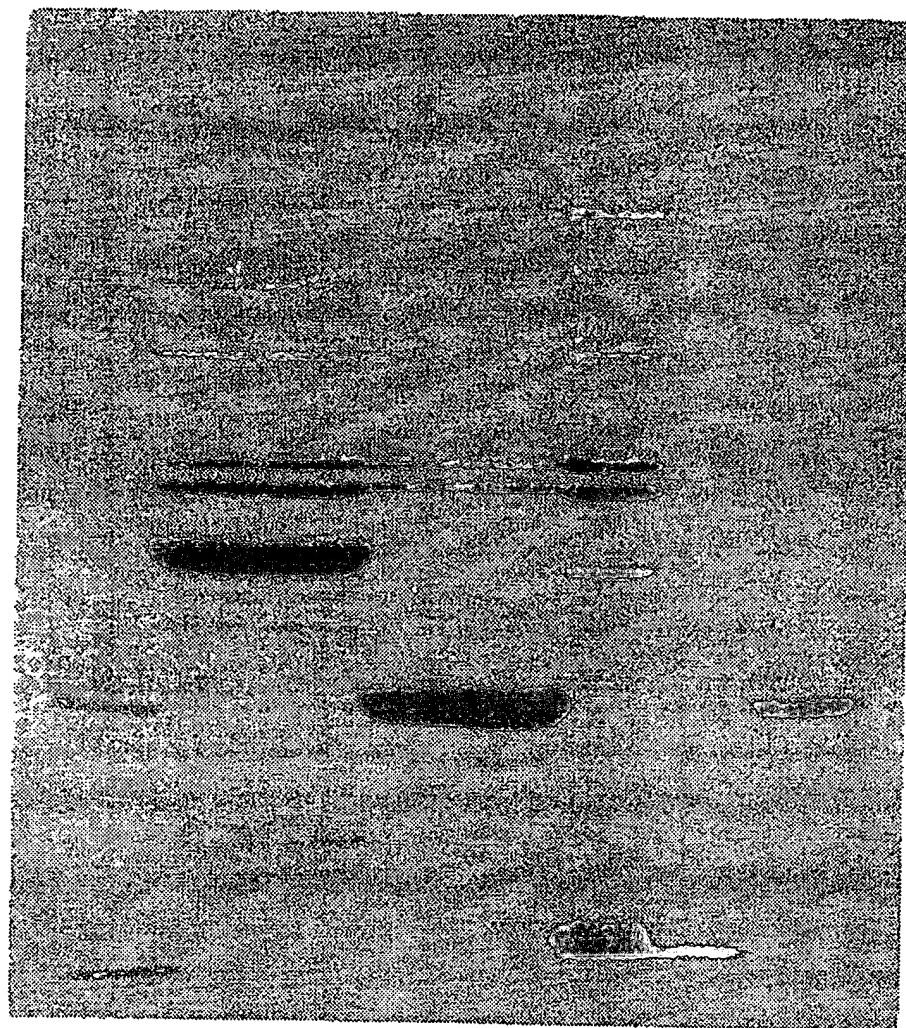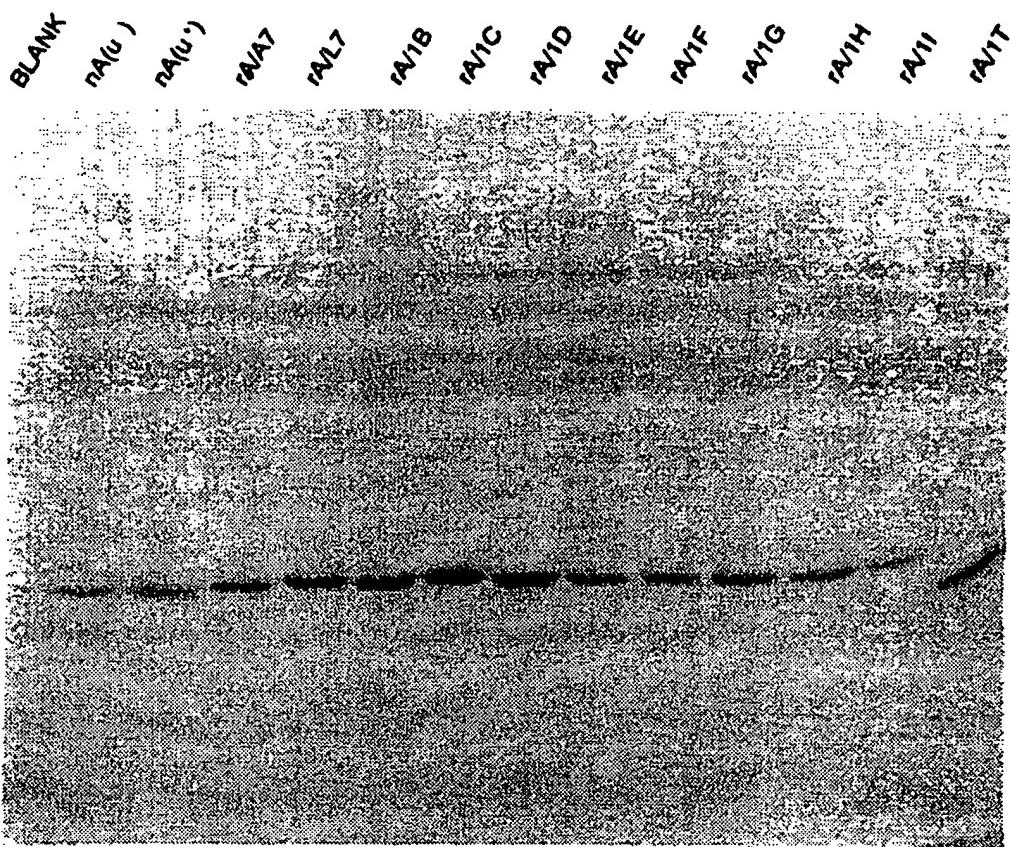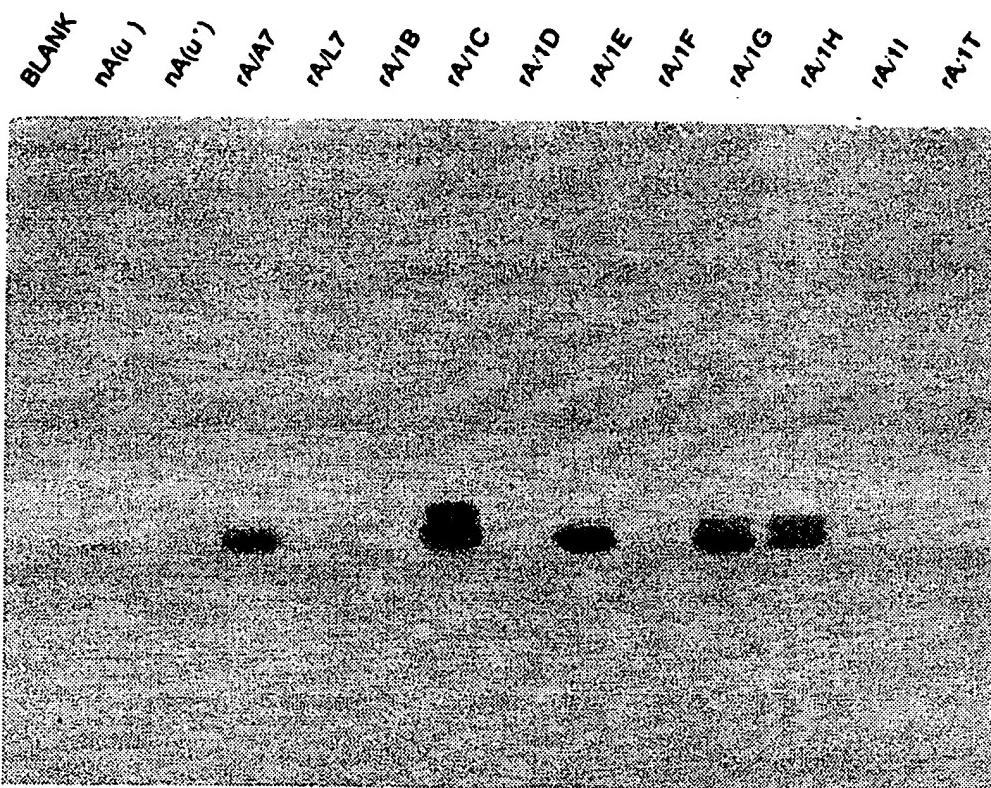
FIG. 3

1 2 3 4 5 6 7 8

FIG. 4A

FIG. 4B

FIG. 4C

FIG. 5A

FIG. 5B

FIG. 5C

FIG. 6A

FIG. 6B

FIG. 7A

FIG. 7B

FIG. 8A

CTX A1 analogs ->

FIG. 8B

Autoradiogram: RBC Membranes					arg 11 his	arg 11 his
B	A	A+u	rA1	ser 10 gly		

Original aa
Position
New aa
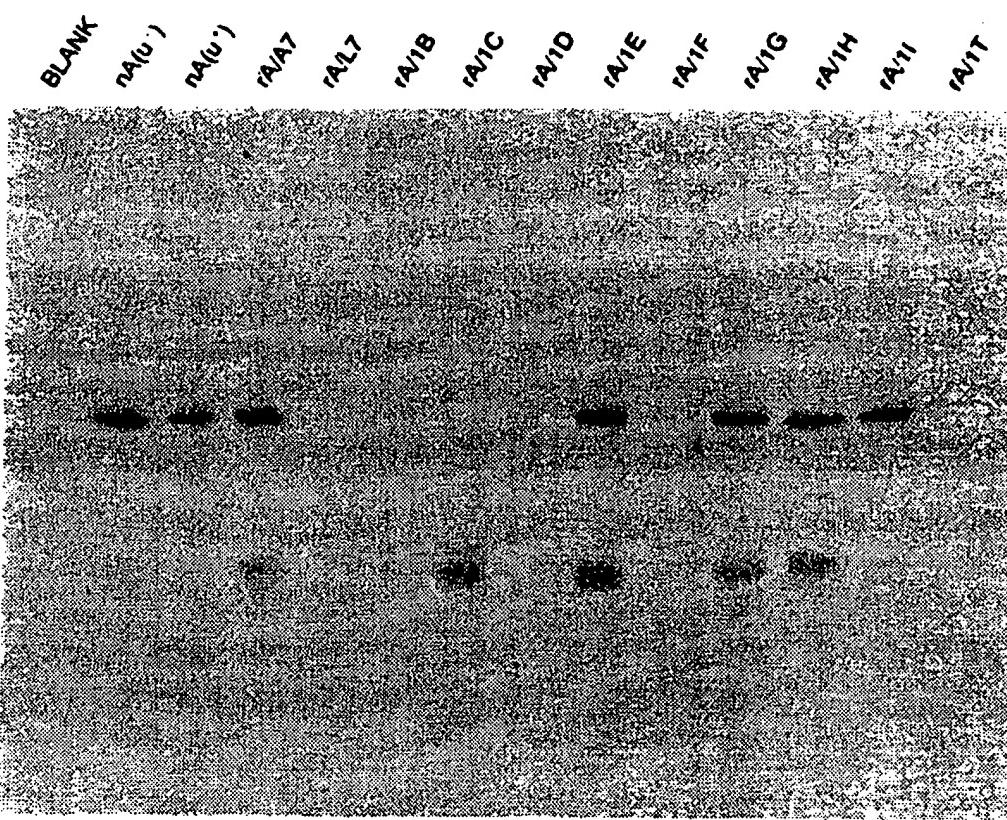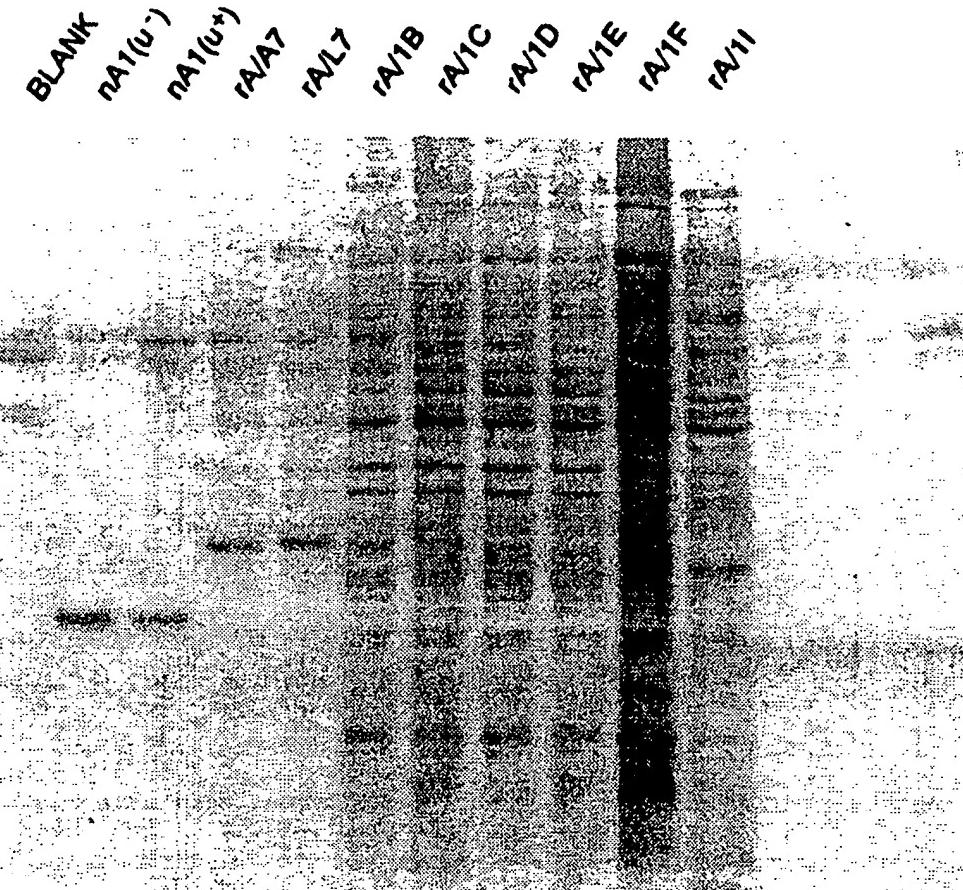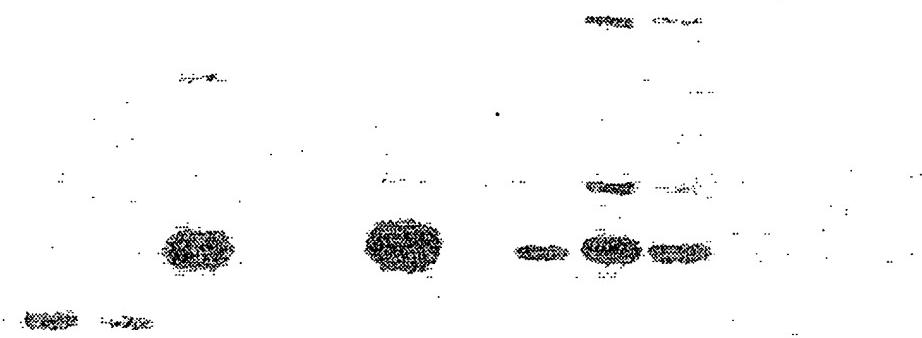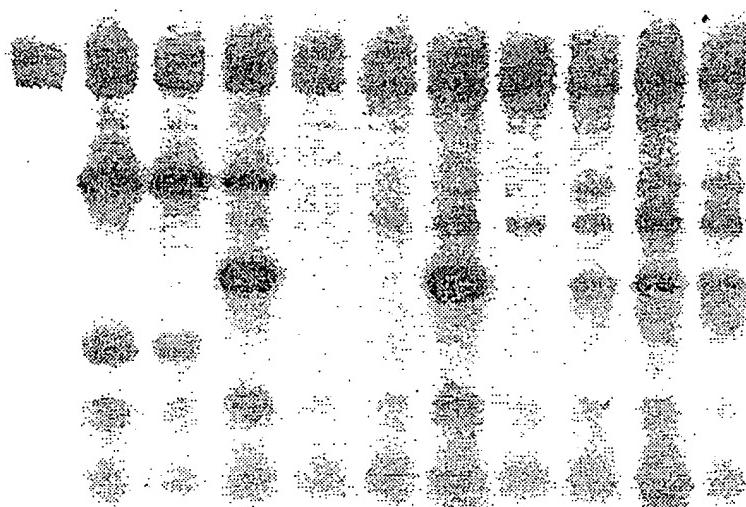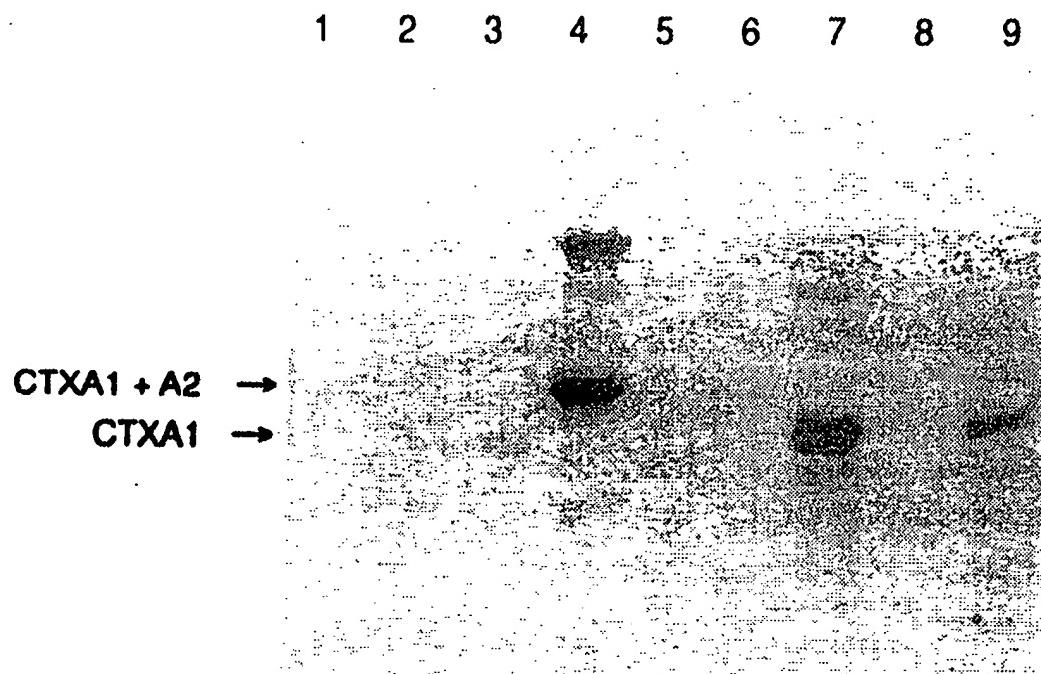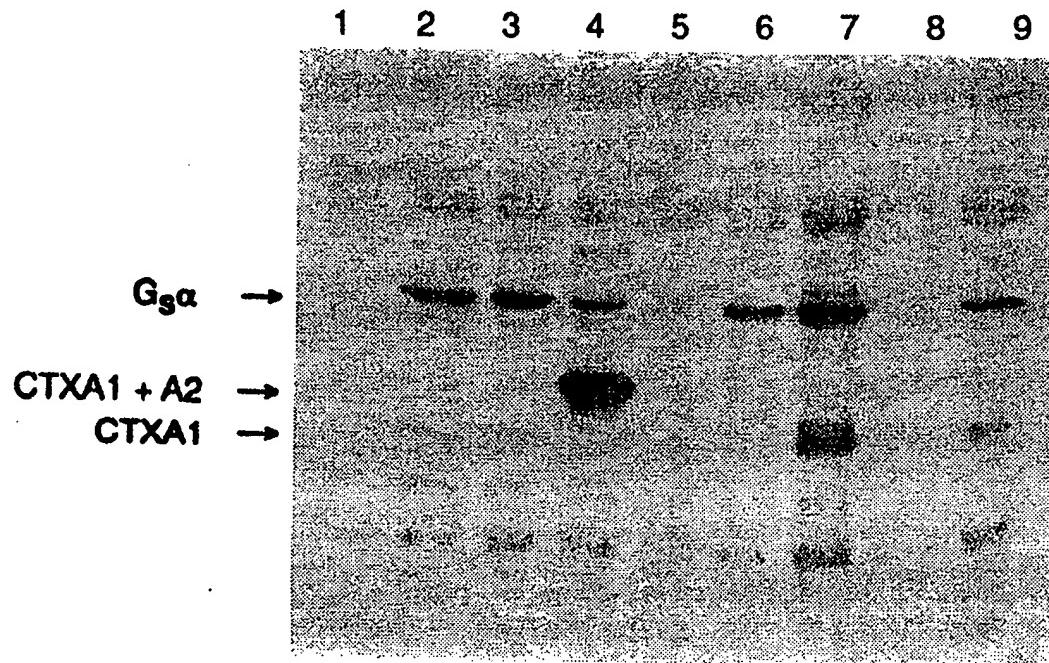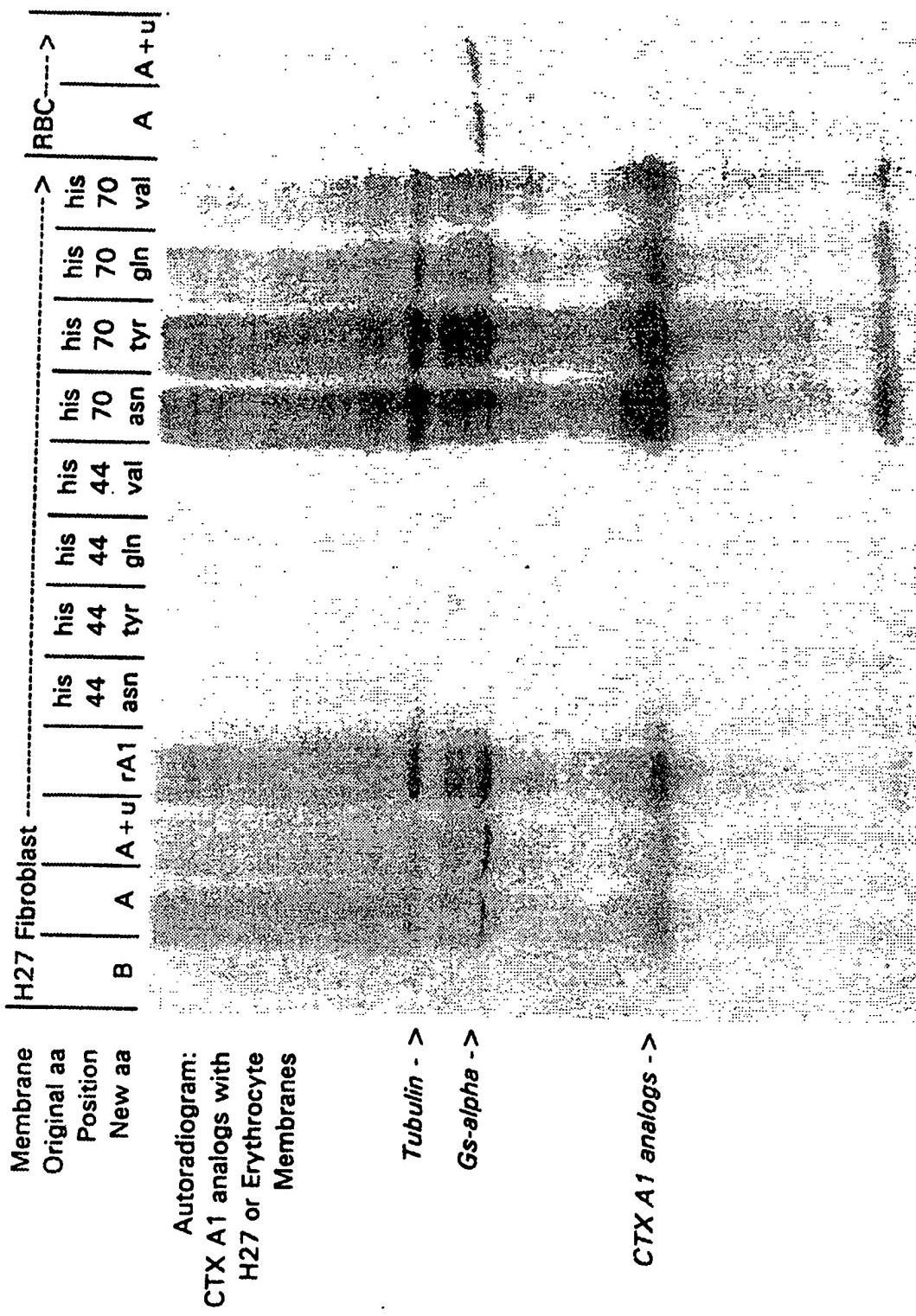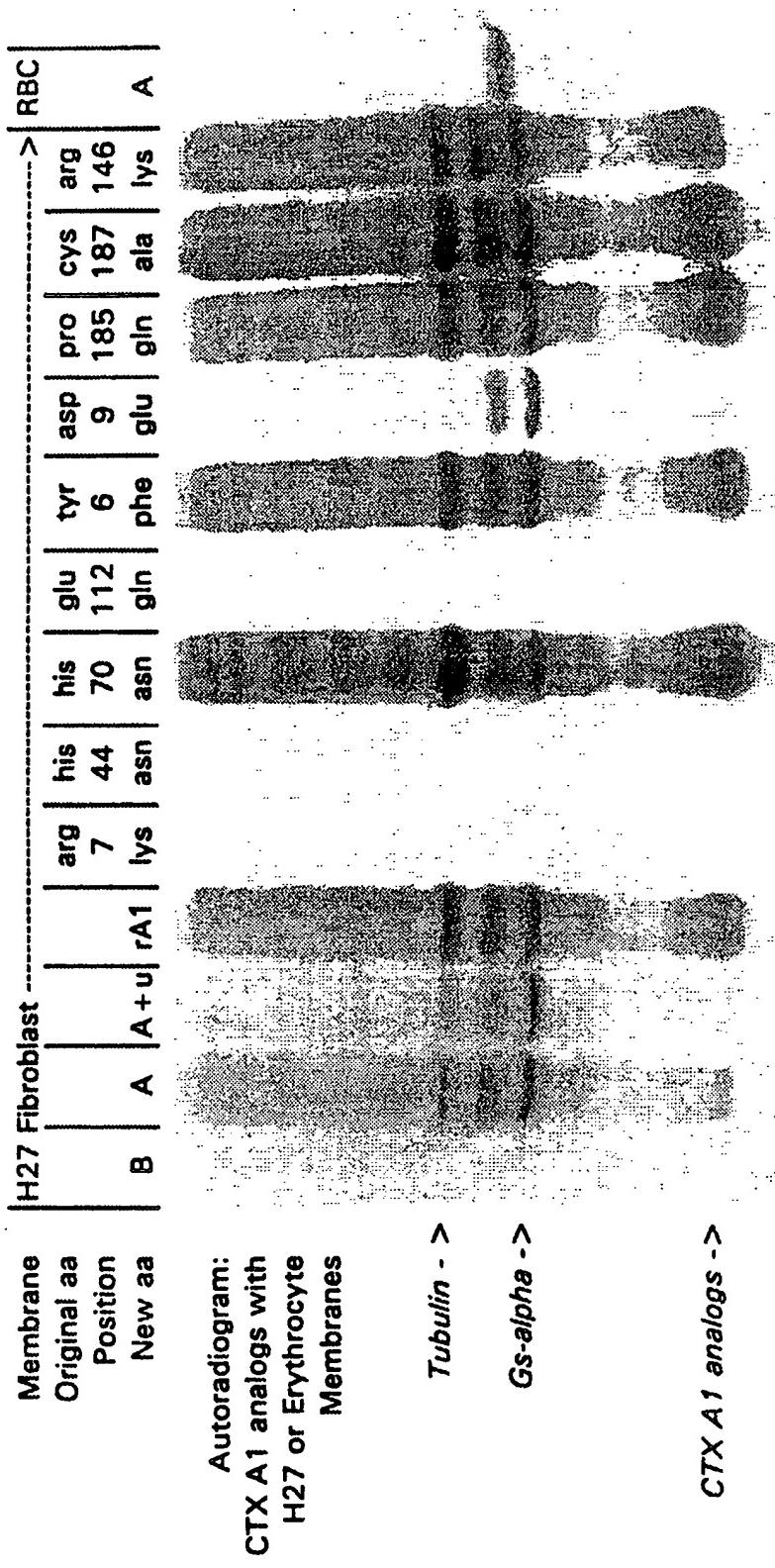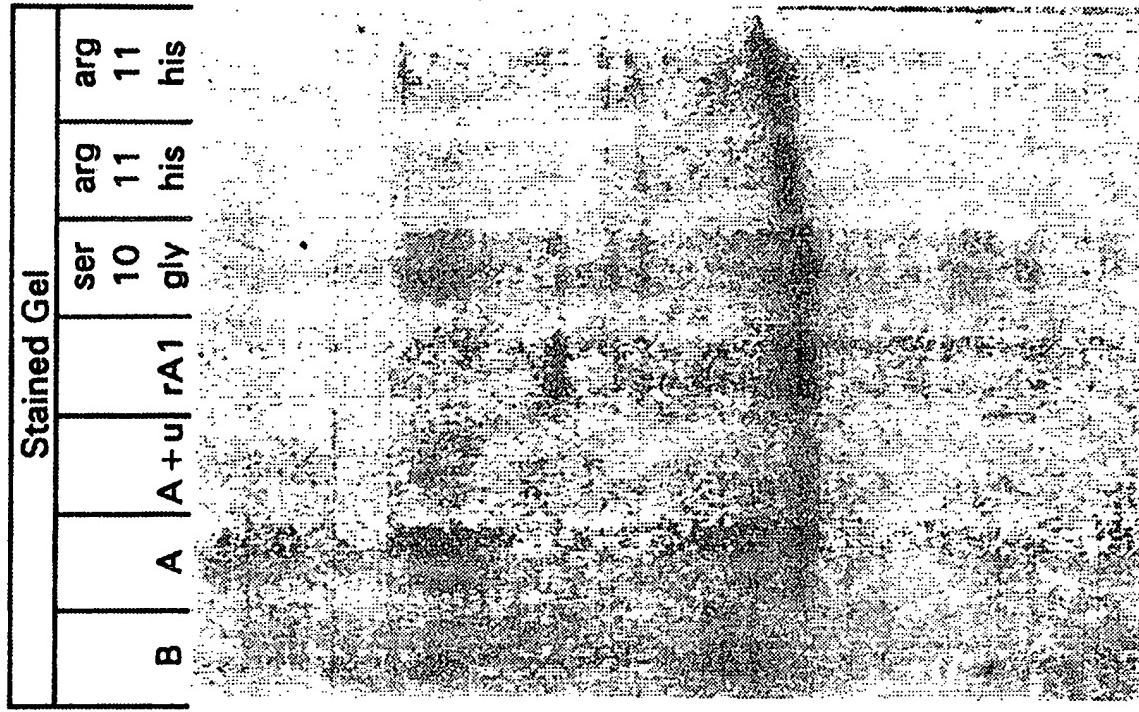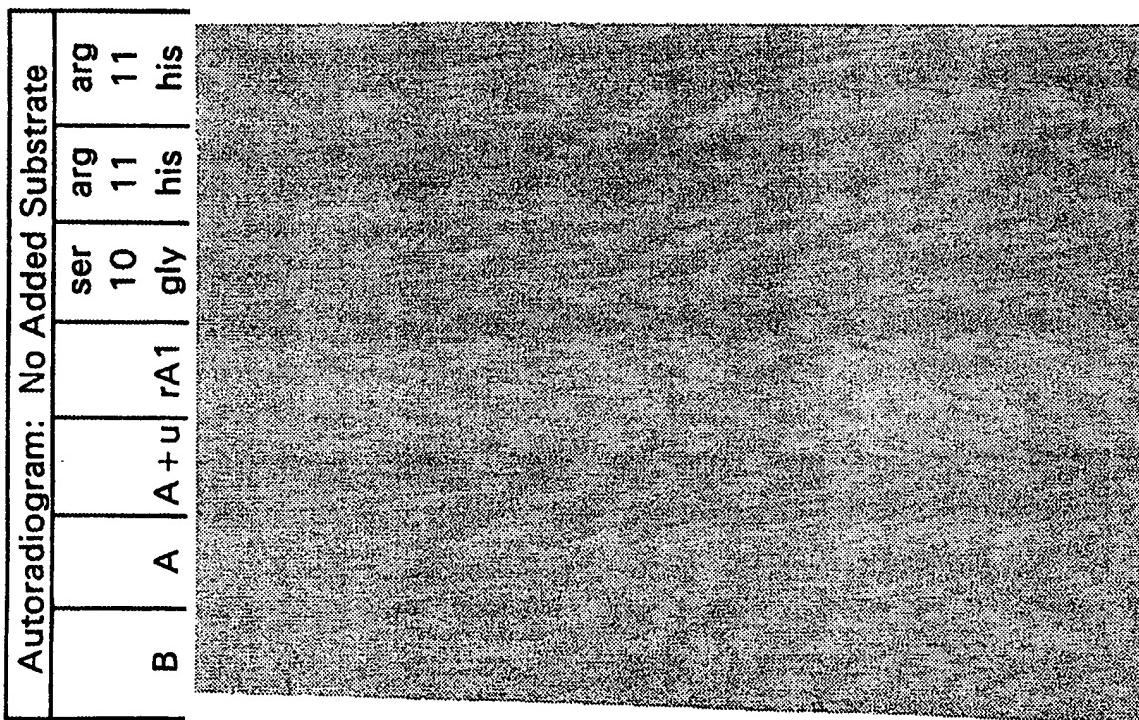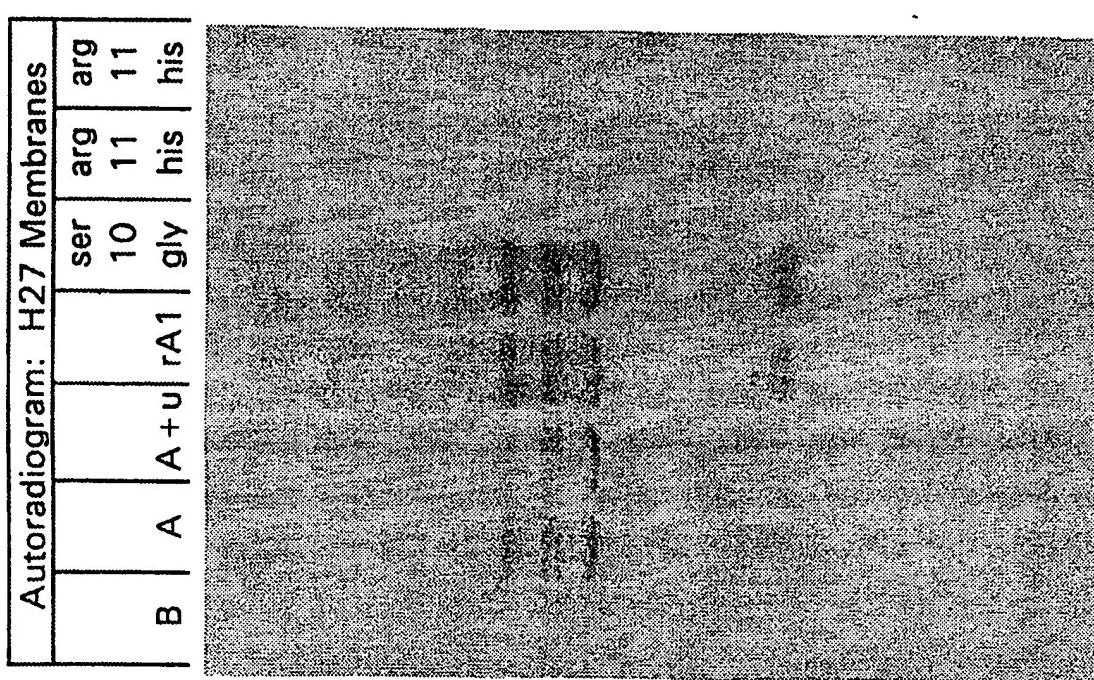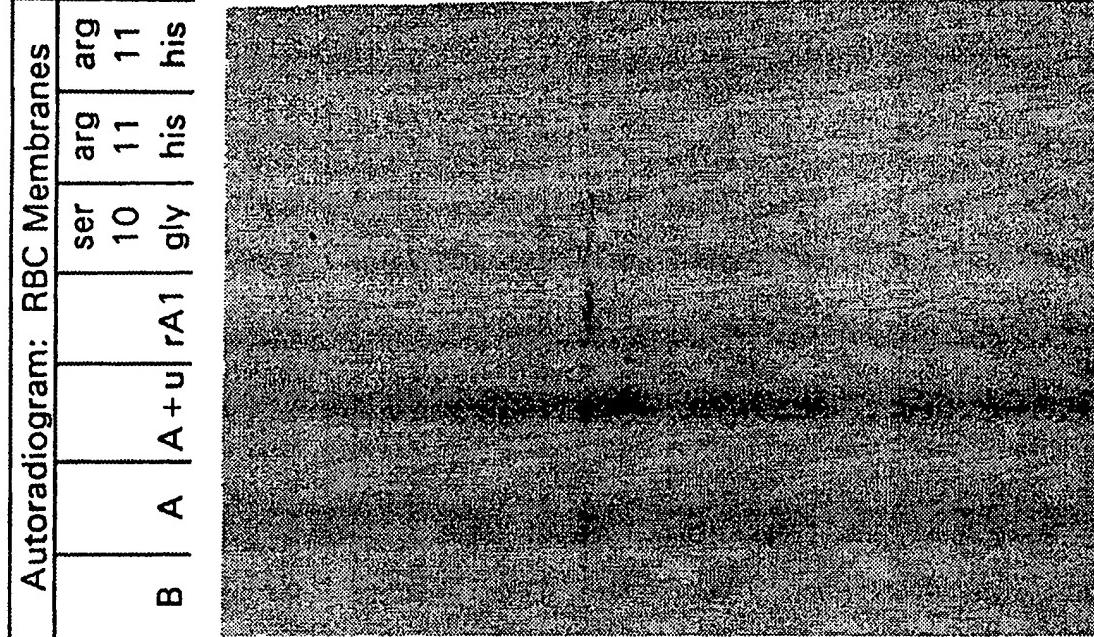
Tubulin ->

Gs-alpha ->

FIG. 8C

Autoradiogram: H27 Membranes					arg 11 his	arg 11 his
B	A	A+u	rA1	ser 10 gly		

FIG. 8D

IPC(5) :A61K 39/106; C12N 1/20, 1/21, 5/10, 9/12, 15/31, 15/09; C12P 21/02
US CL :435/172.3, 194, 240.2, 252.1, 252.3, 252.33; 424/88; 536/27
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/172.3, 194, 240.2, 252.1, 252.3, 252.33, 909, 69.3, 71.1, 172.1; 424/88; 536/27

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE, MEDLINE, BIOSIS
search terms: cholera toxin, reactogenicity, mutant, analog, toxoid

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim N .
X	RESEARCH IN MICROBIOLOGY, Volume 141, issued 1990, J.B. Kaper et al., "Recombinant Attenuated Vibrio cholerae Strains Used as Live Oral Vaccines". pages 901-906, entire document especially pages 903-904.	1-6, 9-11, 13-15, 29, 31, 33
P,Y	CHEMICAL ABSTRACTS, Volume 115, No. 17, issued 28 October 1991, Y. Lobet et al., "Effect of Site-directed Mutagenic Alterations on ADP-Ribosyltransferase Activity of the A-subunit of Escherichia coli Heat-labile Enterotoxin", pages 433-434, abstract no. 178165, Infection and Immunity, 59(9), pages 2870-2879.	1-7, 9-16, 18-24, 26-33
X A	US, A, 4,935,364 (Kaper et al.) 19 June 1990, entire document.	33 1-32
X A	US, A, 4,882,278 (Mekalanos) 21 November 1989, entire document.	33 1-32
X Y	US, A, 4,666,837 (Harford et al.) 19 May 1987, entire document.	34-36 1-33
A	US, A, 4,328,209 (Finkelstein et al.) 04 May 1982.	18-28

X Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing f the international search report
13 August 1992	1 9 AUG 1992

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, f the relevant passages	Relevant to claim N .
P,X Y	CHEMICAL ABSTRACTS, V lume 116, N . 7, issued 17 February 1992, W. Burnette et al., "Site-specific Mutagenesis f the Catalytic Subunit f Cholera Toxin: Substituting Lysine for Arginine 7 Causes Loss of Activity", page 273, abstract no. 53261, Infection and Immunity, 59(11), pages 4266-4270.	<u>1-7, 10-16, 29-30, 33-36</u> 8, 17-29, 31-32
Y	SCIENCE, Volume 232, issued 06 June 1986, C. Locht et al., "Pertussis Toxin Gene: Nucleotide Sequence and Genetic Organization", pages 1258-1264, especially page 1261-1262 paragraphs 1 and 6 and Fig. 3	1-33
Y	SCIENCE, Volume 242, issued 07 October 1988, W.N. Burnette et al., "Pertussis Toxin S1 Mutant With Reduced Enzyme Activity and a Conserved Protective Epitope", pages 72-74, entire document.	1-33